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It is characteristic of Science and Progress that they continually open new fields to our visions.—PASTEUR.

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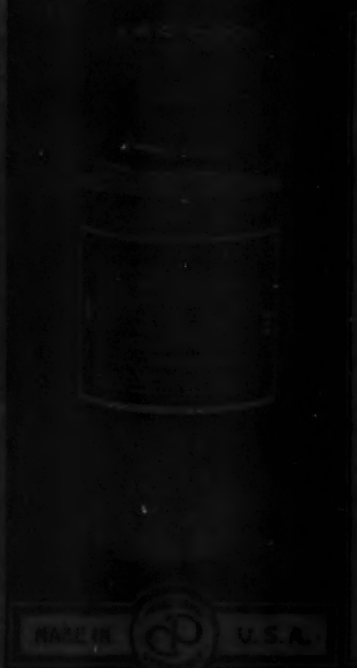

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GELATIN LIQUEFACTION BY BACTERIA

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Department of Chemistry, Iowa State University

Received for publication August 21, 1922

The liquefaction of gelatin is generally recognized and employed as a fundamental criterion for the differentiation of bacterial species. Unfortunately the methods in vogue for observing this property are crude and unreliable. Usually nutrient gelatin is inoculated by stabbing, kept at a temperature below its gelation point, and compared with controls to detect any liquefaction. Measurement of the rate of liquefaction is sometimes attempted by inoculating the entire surface of a tube of gelatin and recording the depth of liquid formed, after different periods of storage, but comparable results are rarely obtained.

The first step in the liquefaction of gelatin is peptization or transformation from the gel to the sol state; from a very viscous to a more fluid condition. Davis and Oakes (1922), in a recent paper on the physical characteristics of gelatin solutions have shown that the transformation from the gel to the sol state in 4 per cent gelatin solution takes place at 38.03°C. Above this temperature, the viscosity remains constant on ageing or decreases if the temperature is sufficiently high to cause hydrolysis. Below this transition point, viscosity increases with age.

At the meeting of the Society of Bacteriologists in 1919, William M. Clark (1920) reported some observations on proteus gelatinase in which he determined the solidification time by a modification of the method of Palitzsch and Walbum. Unfortunately the complete paper has not been published and the abstract does not give full details.

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We feel that further observations on viscosity changes are much needed and would be very instructive. It will be shown in this paper that in autoclaved gelatin, stored at 22°C., the viscosity may change but little for two or three days, after which it increases markedly for at least a week or ten days. Obviously a gelatinolytic organism may bring about liquefaction quite rapidly when inoculated into freshly prepared gelatin, and much more slowly if introduced into the same medium ten days or two weeks later, for in the latter instance more of the gel form must be transformed to the sol state to make liquefaction evident. Some method which could be employed over a wide range of temperature for measuring the rate of liquefaction of gelatin by bacteria is sorely needed.

In this preliminary paper are recorded some observations on the change in viscosity and formol (Sörenson) titration of gelatin subjected to bacterial decomposition.

EXPERIMENTS WITH LOW TEMPERATURE ORGANISMS

Seven organisms isolated from milk were employed. These strains grew very well at room temperature, poorly at 37°C., and not at all at 39 to 40°C. The characteristics of the organisms are indicated in the following table, kindly furnished us by Miss Lulu Soppeland of the Iowa Engineering Experiment Station who will report a more detailed study in the near future.

Medium. The gelatin medium employed consisted of

Peptone (Difco)	1.0 gram
Gelatin (Difco)	35.0 grams
Water (distilled)	1000 cc.

This was heated at 60 to 65°C. till dissolved, then further heated in a double boiler for 15 minutes, the reaction adjusted to pH 8.0, loss due to evaporation made up, and heated an additional 15 minutes, then filtered through cotton and flannel, tubed (25 cc. quantities) and sterilized at 15 pounds for fifteen minutes. The medium was allowed to cool at room temperature and the following day (twenty-four hours) inoculations were made.

LAB. NO.	GENERAL CHARACTERISTICS	S. A. B. GROUP NO.
2019	Gram negative; short rods; singly or short chains; non-motile; spores not found; gelatin not liquefied; greenish pigment on agar	222.3331433
2011	Gram positive; irregular shapes; budding frequent; spores not found, non-motile; gelatin slowly liquefied. (Mold like)	221.2323913
2060	Gram negative; short rods; singly or short chains; spores not found; non-motile; gelatin liquefied slowly; orange pigment	221.3333633
2053	Gram positive; short rods singly or short chains; spores not found; non-motile; gelatin liquefied; orange yellow pigment	221.2323623
1021	Gram positive; short rods singly or short chains; spores not found; motile; gelatin liquefied	221.1112811
1059	Gram positive; short rods; singly or short chains; spores not found motile; gelatin liquefied; yellow pigment	221.2323512
1045	Gram positive; short rods; singly or short chains; non-spore-forming; non-motile; gelatin liquefied; yellow pigment	221.2323513

The inoculum consisted of 1 cc. of a twenty-four-hour broth culture of the test organism and incubation was at 22°C. Control tubes were inoculated with 1 cc. of sterile broth.

Peptone water (0.1 per cent) was similarly inoculated to afford a correction for the peptone in the gelatin medium. Each organism was inoculated into 5 tubes gelatin and peptone water and at stated intervals one tube of each medium was removed from the incubator and examined.

Viscosity measurements were made at 25.6°C. by observing the time of discharge of a given volume in an Ostwald viscosimeter. In the following table the viscosity is expressed in terms of water, at the same temperature, as unity. The time of flow for water was 88.5 seconds.

Formol titrations were carried out in the following manner: To 20 cc. of distilled water in an evaporating dish were added 5 cc. of gelatin (or peptone) medium and the reaction adjusted

TABLE 1

Effect of period of incubation on formol (Sörenson) titration and viscosity

INCUBATION (22°C.)	FORMOL TITRATION				VISCOSITY (25.6°C.) REFERRED TO H_2O
	Peptone	Peptone gelatin	Gelatin	Increase from gelatin	
			Controls		
days					
0	0.13	1.46	1.33*		5.38
1	0.13	1.46	1.33		5.38
3	0.14	1.35	1.21		5.14
5	0.14	1.29	1.15		12.67
7	0.13	1.42	1.29		19.15
12	0.13	1.48	1.35		†
			Organism 2019		
0	0.13	1.46	1.28		5.38
1	0.26	1.51	1.25	−0.03	5.29
3	0.23	1.36	1.13	−0.15	5.21
5	0.26	1.43	1.17	−0.11	9.82
7	0.18	1.60	1.42	+0.14	13.66
12	0.27	1.53	1.26	−0.02	†
			Organism 2011		
0	0.13	1.46	1.28		5.38
1	0.16	1.50	1.36	0.08	3.51
3	0.19	1.70	1.51	0.23	2.91
5	0.26	1.79	1.54	0.26	2.05
7	0.21	2.66	2.45	1.17	1.75
12	0.34	2.73	2.39	1.11	1.27
			Organism 2060		
0	0.13	1.46	1.28		5.38
1	0.17	1.54	1.37	0.09	4.08
3	0.17	1.90	1.73	0.45	3.66
5	0.24	1.88	1.64	0.38	4.02
7		2.11	(1.84)	0.56	3.58
12	0.32	4.28	3.96	2.68	1.29

* Employed 1.28 as the average of controls for correction.

† Too viscous to ascertain.

Formol titration expressed in terms of N/1 NaOH required per 100 cc. of medium for neutralization after treatment with formaldehyde.

TABLE 1—*Continued*

INCUBATION (22°C.)	FORMOL TITRATION				VISCOSITY (25.6°C.) REFERRED TO H_2O
	Peptone	Peptone gelatin	Gelatin	Increase from gelatin	
			Organism 2053		
days					
0	0.13	1.46	1.28		5.38
1	0.17	1.58	1.41	0.13	2.61
3	0.22	1.81	1.59	0.31	2.08
5	0.30	1.91	1.61	0.33	1.92
7	0.25	2.57	2.32	1.04	1.76
12	0.32	2.99	2.67	1.39	1.32
			Organism 1021		
0	0.13	1.46	1.28		5.38
1	0.36	2.24	1.88	0.60	1.80
3	0.37	3.61	3.24	1.96	
5	0.41	4.83	4.42	3.14	1.37
7	0.41	5.78	5.37	4.09	1.38
12	0.50	7.64	7.14	5.86	1.20
			Organism 1059		
0	0.13	1.46	1.28		5.38
1	0.34	2.42	2.08	0.80	1.49
3	0.50	5.99	5.49	4.21	1.35
5	0.79	9.20	8.41	7.13	1.27
7	0.83	13.61	12.78	11.50	1.21
12	0.78	18.18	17.40	16.12	1.16
			Organism 1045		
0	0.13	1.46	1.28		5.38
1	0.27	2.16	1.89	0.16	1.54
3	0.40	4.16	3.76	2.48	1.34
5	0.62	6.45	5.83	4.55	1.34
7	0.72	9.48	8.76	7.48	1.33
12	0.74	14.00	13.26	11.98	1.17

to neutrality to phenolphthalein, 10 cc. of a 50 per cent formalin (neutral to phenolphthalein) was then added, and after ten minutes the sample was titrated with N/50 NaOH. In the tables the formol titration is expressed in terms of cc. N/1 NaOH per 100 cc. of medium or per cent normality of acid liberated by the formaldehyde. Some difficulty was encountered in

determining the exact end point, but in general the differences observed with the various cultures were so great that this end point error (0.1 to 0.15 cc.) may be disregarded.

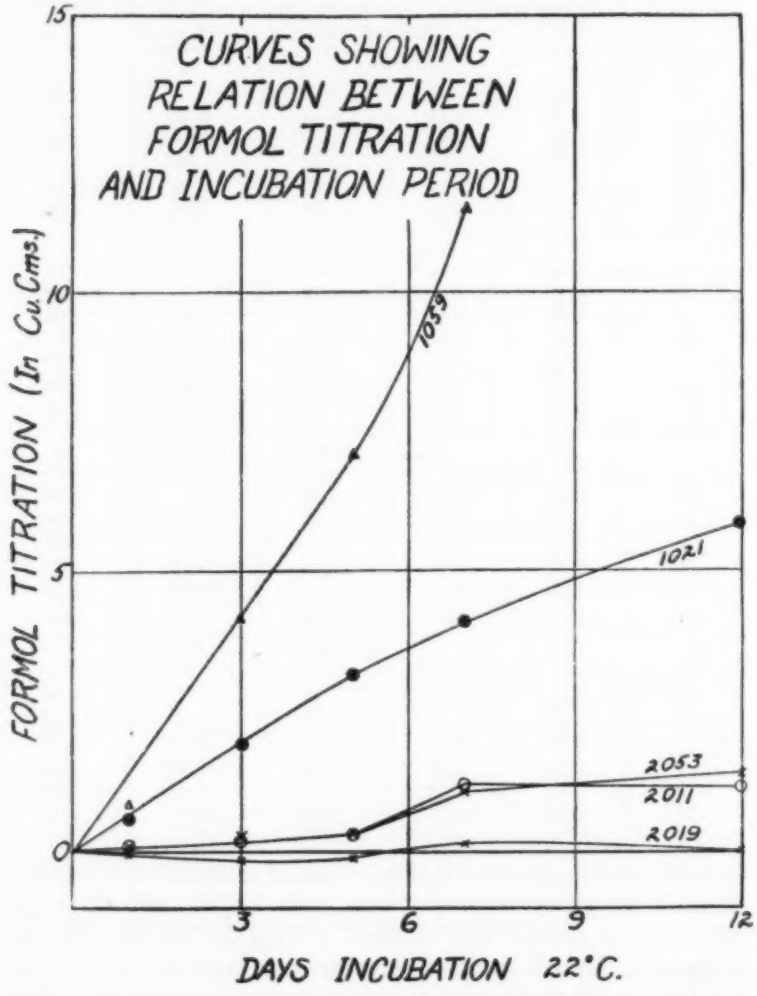

FIG. 1. INCREASE IN FORMOL TITRATION (CC. N/1 NaOH REQUIRED PER 100 CC. MEDIUM)

The results are detailed in table 1, and in figures 1 and 2 are shown the changes in relative viscosity and formol titrations brought about by the growth of the various bacteria studied.

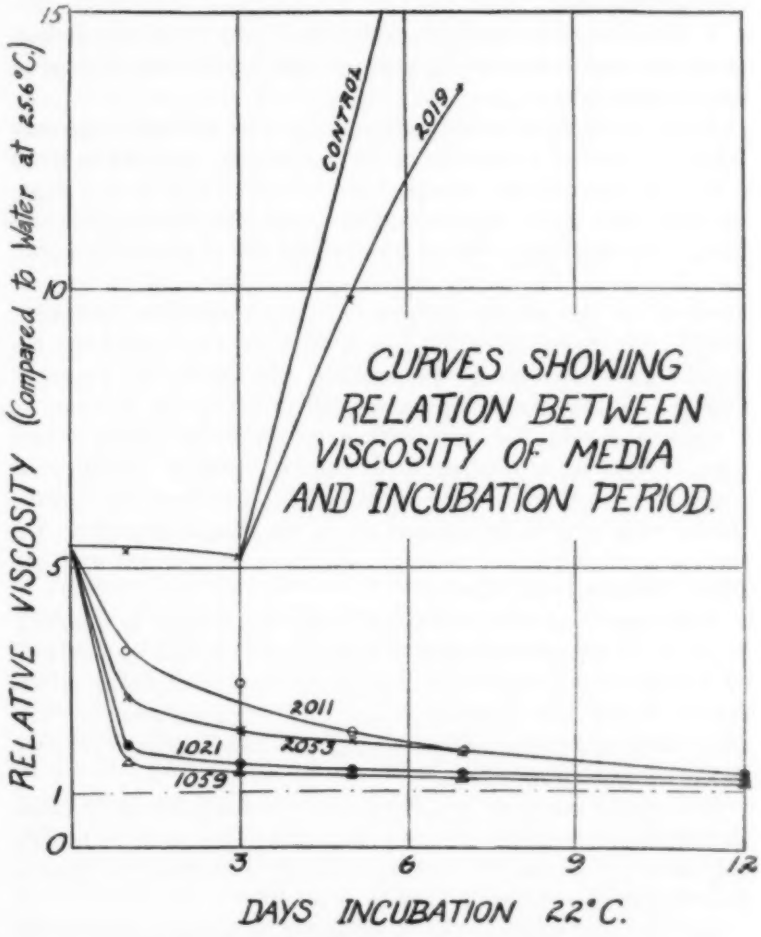

FIG. 2. CHANGE IN VISCOSITY OF PEPTONE-GELATIN

The viscosity and formol titration changes indicate that, with respect to their action on gelatin, bacteria may be subdivided into three groups as follows:

1. Gelatin not hydrolized; liquefaction negative.

2. Gelatin partially hydrolized and liquefied. Subsequent decomposition slow if any; accompanied by slight increase in formol titration.

3. Gelatin more completely hydrolized, and liquefied. Subsequent decomposition, rapid; accompanied by marked increase in formol titration.

As an example of the non liquefying type we have organism 2019. The relative viscosity of the medium (0.1 per cent peptone —3.5 per cent gelatin) increased from 5.38 to 9.38 in five days, to 13.60 after seven days incubation, and after twelve days the gelatin was solidified. There was a slight rise in formol titration, but after correcting for the peptone, the increase, which may be ascribed to the gelatin proper, became a negative quantity. This is due probably to the fact that in peptone water the organism grew throughout the medium whereas in the peptone-gelatin medium growth was restricted to the upper layers.

Organisms 2011, 2053 and 2060 are representative of the second type. There is a distinct drop in the viscosity twenty-four hours after inoculation (from 5.38 to 3.51, 4.08 and 2.62 respectively) with practically no increase in the formol titration. On further incubation the viscosity continues to decrease and the formol titration rises slowly.

With organisms 1021, 1059 and 1045, the change in viscosity is much more marked falling to 1.86, 1.49 and 1.54 respectively in twenty-four hours. The rise in the formol titration is relatively rapid, the increase in one day being as great as was observed in five to seven days among the strains of the previously described liquefying group.

The results are quite in accord with the statement of Berman and Rettger (1918) that "the power of an organism to liquefy gelatin is not necessarily accompanied by the ability to decompose the gelatin and seize upon it as food."

To ascertain whether the liquefaction is independent of the presence of living microörganisms, the following experiment was carried out.

Eight tubes of the above batch of peptone-gelatin which had solidified were heated for thirty minutes at 55°C. to convert to the sol form, and then cooled to 22°C.

To each of four tubes were added 2.5 cc. of 5 per cent phenol (giving a concentration of 0.5 per cent phenol) and to each of the remaining 4 gelatin tubes were added 2.5 cc. of broth. Two of the phenolated and two of the ordinary gelatin tubes were inoculated with 1 cc. of a forty-eight-hour broth culture of organism 1059, and to the remaining (control) tubes was added 1 cc. of broth so as to maintain the concentration of gelatin as equal as possible. All were incubated at 22°C. The results are indicated in table 2.

TABLE 2

Effect of period on incubation and presence of phenol (0.5 per cent) on formol titration and viscosity of peptone gelatin

ORGANISM	INCUBATION (22°C.)	NOT PHENOLATED		PHENOLATED	
		Formol titration	Viscosity	Formol titration	Viscosity
	days				
None	1	1.52	4.95	1.56	3.34
	6	1.54	6.58	1.56	4.90
1059	1	3.04	1.50	1.65*	1.62
	6	10.44	1.18	1.71	1.31

* Corrected for formol titration of 1 cc. broth inoculum (0.17 cc.)

There was no evidence at any time of growth in the phenolated medium. The liquefaction was therefore due to the presence of enzymes, secreted by the microörganisms in question. Considering the formol titration it is clearly evident that in the absence of bacterial growth (phenolated gelatin) there was practically no increase, whereas the increase was very considerable in the non phenolated tube. These results are in accord with those obtained by Kendall and his associates (1922) on studies with *Proteus*. They observed that liquefaction of gelatin by the bacteria-free enzyme was accompanied by a slight increase in ammonia and amino acids (formol titration), whereas in the presence of the viable bacteria, the ammonia content rose very markedly.

The slight increase in the formol titration observed in our experiments with phenolated gelatin is taken to represent the increase in ammonia and amino acids accompanying the enzymic

hydrolysis of gelatin. The marked rise obtained with the actively growing bacteria is most likely due to liberation of ammonia due to intracellular deaminization. Further work on this point is contemplated.

SUMMARY

The change in viscosity of a gelatin medium and simultaneous rate of increase of the "formol titration" was observed with 7 organisms.

The viscosity was found to drop before the formol titration begins to rise. The rate of increase in formol titration serves to distinguish two types of gelatin liquefiers.

A standardized method for ascertaining the change in viscosity of gelatin culture media should be far superior to the present methods of detecting gelatin liquefaction. A temperature of 40°C. which is slightly above the gelation point is suggested as desirable for this purpose and is now under investigation.

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VARIATION IN THE LIMITING HYDROGEN-ION CONCENTRATIONS OF STREPTOCOCCI

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The limiting hydrogen-ion concentration produced by streptococci in fluid media has been suggested as a possible means of differentiating between bovine and human strains. Some authors have called attention to a parallelism between the limiting hydrogen-ion concentration and pathogenicity of streptococcus strains.

HISTORICAL

The literature on the limiting hydrogen-ion concentration of streptococci has been reviewed in another place (Arnold, 1921).

TECHNIC

The strains of streptococci studied were obtained from the throats of diphtheria patients. All cultures were centrifuged well before the hydrogen-ion determination was made. Each strain was plated out twice on blood agar and colonies picked before we began the limiting hydrogen-ion determinations. Hiss-serum water was prepared in the usual manner. For the past three years we have mantained our stock cultures of streptococci on Holman's cooked meat medium (Holman, 1919). All of the media used in this work were made up in large quantities and the same original lot was used throughout the entire course of the experiments.

EXPERIMENTS

Table 1 gives the results of the comparison of the limiting hydrogen-ion concentration of 12 hemolytic and 4 viridans

strains of streptococci on 1 percent glucose Hiss-serum water and 1 percent glucose Huntoon hormone bouillon. The initial reaction of both media at the time of the inoculation was pH 7.45.

Table 2 is a record of the results obtained by using Holman's cooked meat medium as a stock culture medium for the streptococcus strains for a month. At periods of about a week apart

TABLE 1

STRAIN	LIMITING H-ION CONCENTRATION AFTER GROWING TWENTY-FOUR HOURS ON 1 PER CENT GLUCOSE. HISS SERUM WATER	LIMITING H-ION CONCENTRATION AFTER GROWING TWENTY-FOUR HOURS ON 1 PER CENT HUNTOON'S HORMONE BOUILLON	
Hemolytic			
H2H	5.5	5.1	0.4
−H17H	5.5	5.3	0.2
H16H	5.5	5.3	0.2
H3H	5.5	5.15	0.35
−H14H	5.4	5.4	0
H11H	4.7	4.6	0.1
H8H	5.2	5.2	0
H6H	5.2	5.0	0.2
H9H	5.4	5.4	0
−H10H	5.2	5.0	0.2
H14H	5.7	5.3	0.4
−H7H	5.4	5.1	0.3
Viridans			
H10V	5.3	5.0	0.3
H8V	5.4	5.2	0.2
H15B	5.1	5.1	0
H12V.	5.0	5.2	−0.2

the strains were transferred from Holman's meat medium to glucose hormone bouillon and the limiting hydrogen-ion concentration determined after twenty-four hours' growth. These same strains were transplanted every other day during the month on plain hormone bouillon, and the limiting hydrogen-ion concentration was determined after twenty-four hours growth on glucose hormone medium. The object of this experiment was to see what effect the Holman's cooked meat medium might have on the

streptococci in reference to their limiting hydrogen-ion concentration in broth cultures and to see if there were any changes in the hemolytic action of the strains of streptococci—in stock cultures.

To illustrate the variations in the limiting hydrogen-ion concentration of streptococci in wounds, as compared with the sugar fermentation of the strains, we wish to record two streptococci wound flora charts. These are typical examples of wound streptococci flora; the full report, including some 50 cases, will be published later in a clinical journal.

DISCUSSION

The hemolytic streptococci have been found by most investigators to be more constant in their cultural and also in their serological characteristics (Kinsella, 1918; Dochez, Avery and Lancefield, 1919; Tunnicliff, 1920; etc.) than the viridans types of streptococci.

The results recorded in table 2 bear this out as a general rule in regard to their cultural characteristics on the media used. The 2 strains of hemolytic types, D32H and D24H, which differed from the 12 strains in their limiting hydrogen-ion concentration after growth on Holman's cooked meat medium for a month, produced a green zone on plating from this medium, while the same strains transplanted daily on glucose bouillon remained hemolytic when plated at the same time.

The viridans strains are not so constant in their limiting hydrogen-ion concentration as the hemolytic strains. The change from viridans to hemolytic types is more pronounced than the change from hemolytic to viridans types.

If the changes produced in the environment of a streptococcus colony on a blood-agar plate is the result of its metabolic function, it is not surpirsing that with changes of a chemical and physical nature in the medium, one should find a change in the action of the diffusible substances from the growing colony on its surrounding medium.

If a strain of streptococcus is transplanted daily for thirty days on Huntoon hormone bouillon and compared with the

TABLE 2

Limiting hydrogen-ion concentration of streptococci

ISOLATED MAY 1, 1922	1 PER CENT GLUCOSE HORMONE MEDIUM MAY 4, 1922	TRANSPLANTED DAILY ON HORMONE MEDIUM MAY 3 UNTIL MAY 8; ON MAY 8 TRANSPLANTED ON 1 PER CENT HORMONE MEDIUM	ON HOLMAN MEAT MEDIUM MAY 4 TO MAY 8; ON 1 PER CENT GLUCOSE HORMONE BOUILLON MAY 8 TO MAY 9	TRANSPLANTED DAILY ON HORMONE BOUILLON MAY 3 TO MAY 19; ON MAY 19 TRANSPLANTED ON 1 PER CENT GLUCOSE HORMONE MEDIUM	ON HOLMAN MEAT MEDIUM MAY 4 TO MAY 20; 1 PER CENT HORMONE BOUILLON MAY 19 TO MAY 20	TRANSPLANTED DAILY ON HORMONE BOUILLON MAY 3 TO MAY 25; ON MAY 25 TRANSPLANTED ON 1 PER CENT GLUCOSE HORMONE MEDIUM	ON HOLMAN MEAT MEDIUM MAY 4 TO MAY 26; ON 1 PER CENT GLUCOSE HORMONE BOUILLON MAY 25 TO MAY 26	JUNE 3, PLATED FROM 1 PER CENT HORMONE BOUILLON ON BLOOD AGAR. PICK COLONY ON 1 PER CENT GLUCOSE HORMONE BOUILLON JUNE 4 TO JUNE 5	JUNE 3, PLATED FROM HOLMAN MEAT MEDIUM ON BLOOD AGAR. PICK COLONY ON 1 PER CENT GLUCOSE HORMONE BOUILLON JUNE 4 TO JUNE 5	
Hemolytic										
D27H2	5.2	5.1	4.7	5.2	4.7	5.1	4.7	5.2	5.2	
D7H	4.6	4.6	4.8	4.6	5.0	4.5	4.8	4.6	4.6	
D14H	4.9	5.0	4.6	5.0	4.8	4.9	4.8	4.8	4.8	
D15H	5.0	5.0	4.7	4.9	4.7	5.0	4.6	5.1	5.0	
D16H	5.1	5.1	4.6	5.0	4.6	4.9	4.6	5.0	5.0	
D18H	4.8	4.9	4.5	5.0	4.7	5.0	4.6	5.1	5.0	
D22H	4.9	4.9	4.6	4.8	4.5	4.9	4.6	5.0	5.0	
D26H	5.3	5.3	4.8	5.4	4.7	5.2	4.8	5.4	5.4	
D25H	5.5	5.5	5.3	5.5	5.6	5.4	5.5	5.5	5.4	
D31H	5.3	5.3	4.8	5.2	4.8	5.2	4.9	5.3	5.3	
D32H	5.0	5.0	4.6	5.1	4.6	5.0	5.1	4.8	4.9	Changed to viridans
A18H3	5.4	5.4	5.1	5.3	4.9			5.3	5.3	
A14H	5.3	5.3	4.9					5.3	5.3	
D24H	4.8	5.0	4.6			5.0	5.4	5.1	5.4	Changed to viridans

Viridans

D4V2	5.1	5.1	4.6	5.0	4.8	5.1	4.7	5.1	4.6	
D5V	5.3	5.2	4.6	5.0	5.0	4.6	4.6	5.2	4.7	Changed to hemolytic
D6V	4.9	4.8	4.7	5.3	5.0	4.9	4.9	5.2	4.9	
D9V2	5.4	5.3	4.8	5.3	5.1	5.4	4.8	5.2	4.8	Changed to hemolytic
D11V	4.9	5.0	4.6	5.1	5.1	5.4	4.8	5.2	5.0	Changed to hemolytic
D12V2	4.7	4.6	4.6	5.0	5.0	5.3	4.8	4.7	5.2	Changed to hemolytic
D20V	5.0	4.9	4.9					4.8	4.7	Changed to hemolytic
D1V	5.1	5.0	4.6	5.4	5.0	5.1	4.7	5.0	4.8	
A16V	4.7	4.6	4.3	4.6	4.6	4.7	5.0	4.7	4.7	
A9V	4.8	4.8	4.7			4.8	5.1	5.0	5.0	Changed to hemolytic

same strain kept on Holman's cooked meat medium for thirty days without transplanting, the environment of this same strain on the two media differs considerably. Holman's meat medium is an excellent medium for anaerobes as well as aerobes. It is used in this laboratory for preserving stock cultures of anaerobes.

TABLE 3

Scalp wound, 2 days after injury

NUMBER OF STRAINS	FERMENTATION OF			LIMITING H-ION CONCENTRATION
	Lactose	Mannitol	Salicin	
				pH
Hemolytic				
4	+	−	+	5.2 to 5.4
4	+	−	+	4.3 to 4.5
Same wound, six days later, clinically a severe streptococcus infection				
Hemolytic				
6	+	−	+	5.3 to 5.5
Viridans				
2	+	−	+	5.4
2	−	−	−	5.4
Toe wound, two days after injury				
Hemolytic				
4	+	−	+	5.0 to 5.5
1	+	−	−	5.3
3	+	−	−	4.2 to 4.5
Same wound, four days later, clinically a severe streptococcus infection				
Hemolytic				
14	+	−	+	5.0 to 5.5
1	+	−	−	5.3
1	+	−	+	4.3

The morphology of the streptococci, both hemolytic and non-hemolytic, changes on the Holman's cooked meat medium. The individual coccus becomes larger, the chains shorter, the gram stain is not so strongly positive; but these characteristics are lost after plating out on blood agar and growing for one generation in glucose bouillon.

Of the strains isolated from the throats of the diphtheria patients in this series, most of them fermented lactose and salicin, but not mannitol. We have called attention to the sugar fermentation characteristics of strains of streptococci isolated from wounds.

The strains of hemolytic streptococci that are most numerous in inflammatory lesions are the lactose and salicin fermenting types, the *Streptococcus pyogenes* (Holman 1916). This type seem to increase in number more than the other sugar fermenting types. Accompanying this change in the fermentation characteristics of the streptococcus flora in an inflammatory exudate is a change in the limiting hydrogen-ion concentration from pH 4.2 to 4.5 to pH 5.0 to 5.5. We have many strains of streptococci isolated from various sources, that have been on Holman's meat medium, with occasional transplantation, for two years. The sugar fermentation of these strains has not changed during this time. Under these conditions of artificial culture the strains are constant in their sugar fermentation characteristics. Whether there is a change in the sugar fermentation of the strains in an inflammatory area leading to a predominance of the lactose and salicin fermenting types, or whether this particular strain finds in such an environment an ideal culture medium for growth, remains an open question.

The limiting hydrogen-ion concentration is more variable than the sugar fermentation characteristics of the streptococcus. If the limiting hydrogen-ion concentration of a strain, either hemolytic or non-hemolytic, is between pH 4.8 to 5.8, it is probably to be interpreted as indicating that that particular strain has been present in an area where it has come in contact with inflammatory exudates. Further work is in progress as to the pathogenicity for mice of strains, with varying hydrogen-ion concentrations, isolated from wounds of patients with a streptococcus infection.

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COAGULATION AND STERILIZATION OF LOEFFLER'S BLOOD SERUM MEDIA UNDER STEAM PRESSURE

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The coagulation and sterilization of Loeffler's blood serum media has always involved a rather complicated technic with the results usually unsatisfactory in the end. Even with the use of the most modern inspissator, a pure ivory white media free from bubbles and irregularities, and in the end assured of sterility, is seldom obtained outside of the large biological laboratories. The difficulty that has always been encountered is that perfect slants cannot be obtained, through the exposure of the media to too rapid a process of heating or to too high a degree of heat.

This difficulty, however, is entirely overcome by a process of coagulating the media in completely sealed and air tight tubes. In this manner the expansion of the air, and consequent rise of pressure within the tubes individually, will prevent any formation of bubbles or irregularities of slants through expansion of the media. With the media in the tubes thus confined and held in a rigid position, 10 pounds of pressure may be applied without producing any shrinkage of the slants. The coagulation, however, is firm and the color of the media a pure white, with the surface entirely smooth. By employing a little care in the collecting of the blood and thus preventing extensive contamination with highly resistant spore bearing organisms, the whole process of coagulation and sterilization may be carried out in less than thirty minutes. The writer has employed the following process with complete success and satisfaction:

1. A sufficient amount of blood is collected in a sterile container so that the desired amount of serum can be obtained within two or three hours after it has clotted. As soon as possible after

collecting, the blood is set in a refrigerator to prevent the multiplication of such contaminating organisms as may have entered in the process of collecting.

2. After the desired amount of serum has separated from the clot, it is poured off into a sterile flask and centrifuged so as to remove such corpuscles as have been carried out by the serum.

3. The proper proportions of serum and glucose broth is now made up and the media distributed in sterile tubes of uniform length and firmly corked with a no. 1 grade of corks.

4. After this the tubes are placed in a metal rack, as shown in the accompanying illustration (fig. 1) and the cover screwed

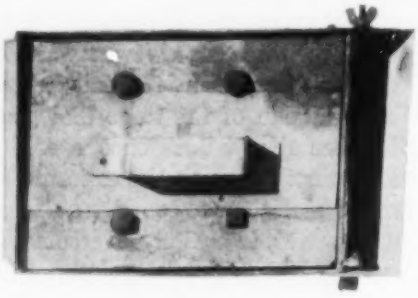

FIG. 1.

down firmly on the corks so as to prevent them from blowing out when the air within the tubes begins to expand as a result of heating.

5. The rack is so constructed that it may be fixed in a tilting position, giving to the tubes any desired slant. After the tubes have been properly packed into the rack and the cover firmly screwed on, the rack is set into the autoclave and the pressure allowed to rise as rapidly as it will under the ordinary amount of heat employed up to 10 pounds, when the heat is turned off and the indicator of the pressure gauge allowed to go back to 0.

6. After the pressure has entirely gone down within the autoclave, the cover is removed and the rack set out in the open to complete the cooling process, which takes some time. When it is certain that the corks will no longer blow out when the cover

of the box is removed, it may be taken off and the process of coagulation and sterilization will be found complete. The media will be of the best quality that can be produced, both in appearance and in actual culture work. An occasional tube may be found at times that still contains an organism that has not been killed. If too much contamination at the time of collecting is suspected, the tubes may be left in the rack with the cover screwed on until the next day when the medium is given another sterilization, the same as on the preceding day.

THE EFFECT OF CERTAIN BACTERIA UPON THE TOXIN PRODUCTION OF BACILLUS BOTULINUS IN VITRO

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Doubtless one of the outstanding problems of anaerobic bacteriology relates to the distribution of obligate anaerobes in nature. In August, 1920, we began a study of the distribution of the toxin forming anaerobes, by testing the toxicity of glucose broth filtrates from impure cultures. Failing to detect any toxin in the cultural filtrates from 24 specimens of soil and 10 of human feces, we were led to investigate, first, the methods suitable for such determinations, and second, the effect of microbic association upon toxin production by *B. botulinus*.

THE DISTRIBUTION OF B. BOTULINUS IN NATURE

Van Ermengem (1897) was the first to isolate *B. botulinus* from poisonous food, and its frequent recovery since from a wide variety of food products raises the question of its origin in them. The first record of *B. botulinus* from a source other than food or victim is that of Kempner and Pollack (1897), who came to believe that this organism sometimes inhabits the intestines of healthy swine, which explained the fact that botulism was then chiefly associated with pork products, particularly sausages.

Dickson (cited by Burke, 1919) failed to demonstrate botulinus toxin either in the intestinal contents of 250 grain-fed hogs from the abattoirs of South San Francisco, California, or in 10

[1] Aided by grants from the National Canners Association to the Hooper Foundation for Medical Research, and from the Board of Research at the University of California in 1920–21 and 1921–22.

samples of manure from 2 separate groups of garbage-fed hogs. Hence, it seems unlikely that this organism is a normal intestinal inhabitant in hogs of this region; but the results about to be reported justify further examinations.

During the summer of 1918, Burke (1919) obtained botulinus toxins from 2 out of 9 mixed cultures in glucose broth of bruised and bird-pecked cherries, from 1 out of 5 of hog manure, from 1 out of 3 of pole string beans spotted by insects, and from 1 of a small spider—collected from 5 localities in California, at 4 of which botulism had occurred during the previous winter.

Meyer and Geiger (1921) demonstrated botulinus spores upon market vegetables in San Francisco, and in two manured soils where canned beans had been grown and later caused limberneck in chickens. Since then Dr. Meyer[2] has shown an exceedingly wide distribution of *B. botulinus* in soils.

PROCEDURE FOR CULTURING FECES AND SOILS

Of the soil samples, 1 gram was weighed aseptically and transferred to a constricted tube (Hall, 1915) containing 20 cc. of 1 per cent glucose broth previously boiled five to ten minutes to drive out oxygen. Of the fecal samples, 1 cc., if liquid, or a lump the size of a pea, if solid, was used for inoculation. By gently rolling the tube, as much of the sample as possible was manipulated under the marble seal.

The cultures were incubated at 37°C. for seven to eleven days. They invariably showed abundant growth as indicated by gas and turbidity and were then filtered through sterile Berkfeld filters. The filtrates were preserved on ice to prevent deterioration.

A sterility test of each filtrate was made immediately after filtration by adding 1 cc. to a constricted tube of glucose broth. If no growth occurred during four days' incubation at 37°C., the filtrate was regarded as sterile. In a few instances contaminations developed because of defective filters; these filtrates were refiltered before testing for toxicity.

[2] Personal communication.

Toxicity tests were made by subcutaneous injection of guinea pigs. But, as stated in the beginning, none of the filtrates from 10 specimens of feces and 24 of soil was toxic. And since *B. tetani* and *B. botulinus* were well known to produce strongly toxic filtrates in *pure* glucose broth cultures in the constricted tube the failure to detect toxin in cultural filtrates from so many soils, some at least of which, being fertile, might be presumed to contain *B. tetani*, suggested some defect in our method. The distribution problem was therefore abandoned temporarily in order to determine what the factors of error were by testing for tetanus and botulinus toxins in filtrates of soil cultures inoculated with known strains. This paper presents the experiments upon *B. botulinus* only.

PURE AND IMPURE SOIL CULTURES OF B. BOTULINUS IN VARIOUS MEDIAS AND CONTAINERS

In the following experiments, the procedures already described for culturing samples and securing filtrates were used unless otherwise stated. Our *B. botulinus* 8A, (see Hall, 1922) was used.

Of the toxic filtrates an approximate determination of the minimum lethal dose was made. Each guinea pig was given an initial subcutaneous injection of 1 cc. of filtrate diluted 1:1000. If no symptoms had developed by the fourth day, the dose was increased 10 times, and if this proved to be non-toxic it was increased 10 times again. Long experience in immunizing animals to the toxins of tetanus and botulism teaches that no appreciable error is involved in making rough determinations of toxicity by this method, and it has the advantage of economy in animals. It was not possible always to use guinea pigs of constant weight, but a careful record of the weights was kept, in order to enable us to account for discrepancies attributable to this factor.

Autopsies were made of all guinea pigs that died. Heart blood cultures were planted in constricted tubes of glucose broth, and cultures were made of all visible lesions. No deaths could be attributed to bacterial infections, except in a few instances where

death resulted from a streptococcus epizootic among the guinea pig stock. In these cases, new guinea pigs were injected with the same dose of filtrate; only those animals dying specifically from botulism alone were counted.

THE EFFECT OF GLUCOSE IN THE MEDIUM. SOIL CULTURES OF B. BOTULINUS IN GLUCOSE BROTH AND PLAIN BROTH

The media for this series of experiments were 1 glucose broth and plain broth adjusted by adding an excess of $MgCO_3$, boiling, and filtering out the undissolved residue. The reaction of such a medium is about pH 8.4. The following diverse soils were chosen.

Soil No. 9—clay—Contra Costa County, California.
Soil No. 12—sandy loam—Tulare County, California.
Soil No. 13—gravelly adobe—Tulare County, California.
Soil No. 14—gravelly loam—Glen County, California.

Each soil was cultured in plain and glucose broth in constricted tubes and inoculated with a large loopful of a deep brain culture of *B. botulinus*. Pure cultures of *B. botulinus* No. 8A in plain and glucose broths controlled each set. After incubating the cultures at 37°C. for eight to eleven days, their filtrates were tested for toxicity with the results shown in table 1.

It is clear from the table that in glucose broth there was either inhibition of growth, inhibition of toxin production, or destruction of toxin in the soil cultures. There was no clear-cut evidence either for or against inhibition of growth of *B. botulinus;* one would assume that growth of an anaerobe in the presence of aerobes and of the added particles of soil would be enhanced. The results seemed due rather to inhibition of toxin production or to toxin destruction.

In plain broth the pure control filtrate was toxic only in a dose of 1 cc.; curiously also were 2 of the soil filtrates, but the other 2 killed in doses of 0.01 cc. *B. botulinus* in pure culture grows poorly in mediums without fermentable carbohydrates and rarely produces strong toxin, but the above experiment and

others yet to be described show that under certain conditions of association with aerobes in plain broth stronger toxin is produced than in pure cultures.

TABLE 1

The effect of glucose in soil cultures of B. botulinus

Plain broth

SOIL SAMPLE	WEIGHT OF GUINEA PIG	NON-FATAL DOSE	WEIGHT OF GUINEA PIG	FATAL DOSE
	grams	*cc.*	*grams*	*cc.*
9	540	1.0	—	—
12	560	1.0	—	—
13	280	0.001	460	0.01 (10)
14	340	0.001	410	0.01 (5)
Control (pure)	600	0.1	600	1.0 (4)

Date of culturing 10/1/20—incubation period 11 days.

( ) with number indicates days necessary to kill.

Some soil cultures in plain broth with *B. botulinus* are non-toxic; others are more toxic than the pure control.

Glucose broth

SOIL SAMPLE	WEIGHT OF GUINEA PIG	NON-FATAL DOSE	WEIGHT OF GUINEA PIG	FATAL DOSE
	grams	*cc.*	*grams*	*cc.*
9	290	0.01	450	0.1 (4)
12	370	1.0	—	—
13	220	0.01	430	0.1 (3)
14	260	1.0	—	—
Control (pure)	330	0.0001	440	0.002 (6)

Date of culturing 9/28/20—incubation period eight days.

( ) with number indicates days necessary to kill.

Soil cultures in glucose broth with *B. botulinus* are either non-toxic or much less toxic than the pure control.

THE TIME FACTOR IN PRODUCTION OF BOTULINUS TOXIN

Considering the unavoidable difference in incubation periods in the above experiment, we next tested the effect of varying incubation periods upon the strength of the toxin, secured from pure cultures.

Four constricted tubes of plain broth and four of 1 per cent glucose broth were inoculated with a large loopful of a deep

brain culture of *B. botulinus*. One tube of each series was removed after each three day period of incubation, filtered and tested for toxicity, with the results shown in table 2.

None of the filtrates in the plain broth series was more than weakly toxic. The three day culture was the most active and there was a gradual decrease in toxicity in the six and nine day cultures, while the filtrate of the twelve day sample was non-toxic in a dose of 1 cc. With glucose broth there was a gradual increase in toxicity up to the twelfth day.

TABLE 2

Time factor in production of botulinus toxin by pure cultures

INCUBATION PERIOD	WEIGHT OF GUINEA PIG	NON-FATAL DOSE	WEIGHT OF GUINEA PIG	FATAL DOSE
		Plain broth		
days	*grams*	*cc.*	*grams*	*cc.*
3	500	0.1	170	1.0 (2)
6	360	0.1	300	1.0 (3)
9	400	0.1	400	1.0 (4)
12	330	1.0	—	—
		Glucose broth		
3	430	0.001	490	0.01 (9)
6	—	—	300	0.01 (4)
9	—	—	460	0.001 (6)
12	—	—	530	0.001 (7)

Date of culturing 11/6/20.

( ) with number indicates days necessary to kill.

Only weak toxins were found in plain broth, and the strength decreased slightly with prolonged incubation; much stronger toxins were formed in glucose broth, and the strength increased at 37°C. up to nine days of incubation.

It is clear that glucose is favorable to toxin production in pure cultures of *B. botulinus* and that the time factor was not responsible for the differences between the glucose broth and plain broth filtrates of the pure cultures in our first experiment.

We then took an inventory of methods for detecting botulinus toxin in mixed cultures. Burke (1919) had used glucose broth under oil; we had been unsuccessful, or at least only partially successful, in using glucose broth in constricted tubes with soil

cultures heavily seeded with *B. botulinus*, notwithstanding pure cultures produced strong toxin under these conditions. Dr. K. F. Meyer told us that he was having success in the use of a 0.5 per cent glucose broth medium containing chopped meat in a strong bottle of about 100 cc. capacity fitted with a rubber stopper carrying a cotton plugged glass tube for connection with a suction pump. After evacuating for about fifteen minutes the tube was hermetically sealed in the flame. This method differed from the one that we had tentatively adopted, not only in providing a medium of higher buffer capacity in the chopped meat, but also in the exclusion of obligate aerobic bacteria.

We decided to determine which of these factors was responsible for our failure.

USE OF DIFFERENT MEDIA IN THE VACUUM BOTTLE

Comparative studies were made on *B. botulinus* in 1 per cent glucose broth and 0.5 per cent glucose meat mash medium with and without soil no. 9, which was used in these tests in the bottles. There were about 50 cc. of medium in each bottle, and half of the volume was meat in the chopped meat medium. The cultures were incubated at 37°C. for one week. Both the pure and soil cultures of *B. botulinus* in the meat mash medium proved very toxic, the minimum lethal dose of each filtrate being 0.001 cc. The filtrate from the soil culture in glucose broth was also toxic, with a minimum lethal dose of 0.01 cc. indicating that the inhibition of obligate aerobes might be one factor in the success of this method. No growth occurred in the pure glucose broth culture, owing possibly to imperfect anaerobiosis.

USE OF DIFFERENT MEDIA IN THE CONSTRICTED TUBE

A similar experiment was conducted using the constricted tube with plain broth and plain meat mash medium. The tubes were boiled before inoculating.

As shown in table 3 the filtrates were non-toxic in the pure and only weakly toxic in the soil broth cultures. However, in the meat mash medium, the filtrates from both pure and soil

cultures were very toxic, 1 cc. of a 1:10,000 dilution killing guinea pigs in three days.

These experiments caused us to conclude that the inhibition of obligate aerobes in the vacuum bottle is not the only factor in the success of this method; the buffer nature of the meat mash medium plays perhaps an even more important rôle, for the constricted tube which proved equally successful when used with this medium does not inhibit aerobic growth above the seal. Botulinus toxin can be detected in filtrates from mixed soil cultures in a meat mash medium with the constricted tube,

TABLE 3

Comparative studies on meat digest medium and plain broth in the constricted tube in pure and soil cultures of B. botulinus

CULTURE	MEDIUM	WEIGHT OF GUINEA PIG	FATAL DOSE	
		grams	*cc.*	
Pure *B. botulinus*	Plain broth	—	—*	
Soil 9 + *B. botulinus*	Plain broth	440	0.1	(3)
Pure *B. botulinus*	Meat mash	260	0.0001	(3)
Soil 9 + *B. botulinus*	Meat mash	250	0.0001	(3)

* 1 cc. failed to kill a guinea pig of 790 grams weight.
( ) with number indicates days necessary to kill.
Note the great toxicity of the filtrates from meat mash medium.

and the earlier failure to detect it in filtrates from cultures in glucose broth cannot be blamed upon the type of container used, but rather upon the medium, which fails under the peculiar conditions of the test, although pure cultures of *B. botulinus* produce strong toxin in it.

THE EFFECT OF GLUCOSE IN THE MEAT MASH MEDIUM

We then undertook to determine the difference, if any, due to glucose in meat mash in constricted tubes with pure and soil cultures of *B. botulinus*, also, to see if the absence of the marble seal affected toxin production in either pure or soil cultures.

The meat mash medium was made according to the following formula:

(a) 1 pound of ground meat per liter of water
(b) 0.5 per cent NaCl
(c) 2 per cent Bacto-peptone
(d) Adjusted to pH 7.2 with NaOH
(e) Sterilized in the Arnold sterilizer on three successive days

TABLE 4

Effect of glucose in a meat mash medium in the constricted tube

CULTURE	MEDIUM	MARBLE SEAL	WEIGHT OF GUINEA PIG	FATAL DOSE	
			grams	cc.	
B. botulinus 8A	Plain meat mash	Present	600	0.001	(4)
	1 per cent glucose meat mash	Present	650	0.001	(4)
	Plain meat mash	Absent	720	0.01*	(3)
	1 per cent glucose meat mash	Absent	550	0.001	(10)
B. botulinus plus soil 9	Plain meat mash	Present	610	0.0001†	(65)
	1 per cent glucose meat mash	Present	350	0.0001	(5)
	Plain meat mash	Absent	640	0.001	(6)
	1 per cent glucose meat mash	Absent	690	0.01	(2)‡

Date of culturing 8/25/21—incubated at 37°C., seven days, on ice one week.
* 0.001 cc. had failed to cause symptoms in this guinea pig.
† Very sick with chronic botulism for two months before death.
‡ No more filtrate to test.

Eight constricted tubes were filled with 20 cc. of this medium, with or without 1 per cent glucose, added as indicated, the ground meat occupying about one-half of the space occupied by the liquid in each tube. Four tubes were inoculated with 1 gram of soil 9, as shown in table 4, and all with *B. botulinus*. After incubating at 37°C. for seven days, the cultures were kept on ice for one week before filtering, awaiting the arrival of filters.

Stronger toxins were obtained with than without marble seal in both pure and soil cultures of *B. botulinus* 8A in plain and 1 per cent glucose meat mash medium, owing perhaps to the

more strictly anaerobic conditions with the seal. It was apparent, however that the ground meat helped to exclude oxygen since no growth of anaerobes in pure culture occurred in constricted tubes without seals in clear media unless the depth were increased greatly.

With the marble seal the factor of added glucose in soil cultures in the meat mash medium could be disregarded; equally strong toxins were produced in its absence. Of course the meat contained some muscle sugar. With pure cultures of *B. botulinus* without the seal there was a slightly stronger toxin in 1 per cent glucose meat mash than in plain meat mash.

Possible differences between plain and glucose soil cultures without seals could not be determined, owing to the slight quantity of the last filtrate available for testing. The soil cultures in both plain and glucose meat mash medium, with or without the marble, resulted in stronger toxins than in the corresponding pure cultures.

These experiments proved that the inhibitive factor in our earlier attempts to demonstrate strong botulinus toxin from soil cultures in constricted tubes of glucose broth might be overcome through the use of more heavily buffered mediums. In fact, either plain or glucose meat mash, with or without the marble seal, can be used for detection of *B. botulinus* in soil samples.

STERILE SOIL CULTURES OF B. BOTULINUS

Dr. C. B. Lipman of the Soil Chemistry Department of this university suggested that soils might inhibit the toxin production of *B. botulinus* by chemical action.

Samples 9, 12, 13, and 14 were sterilized in the autoclave for one hour at 18 pounds pressure. One gram amounts of each were placed below the marble in constricted tubes of 1 per cent glucose broth. These tubes were sterilized in the autoclave for one-half hour, after which they were incubated at 37°C. for forty-eight hours. Since no growth appeared during this period, they were regarded as sterile. Each soil culture was then

inoculated by loop from a deep brain culture of *B. botulinus*, along with a control in glucose broth without soil. The cultures were incubated eight to eleven days, and grew actively. They were then filtered and tested for toxicity, with the results shown in table 5.

It is evident that there may have been some slight inhibitive or destructive effect upon the toxin. All the soil cultures gave weaker filtrates than the control, but the sterile soil cultures were considerably stronger than any secured in glucose broth in constricted tubes with non-sterile soils. It is possible that inhibitive substances were destroyed or volatilized by heating.

TABLE 5

The effect of sterile soils in glucose broth cultures by B. botulinus

SOIL SAMPLE	WEIGHT OF GUINEA PIG	NON-FATAL DOSE	WEIGHT OF GUINEA PIG	FATAL DOSE
	grams	*cc.*	*grams*	*cc.*
9		*	700	0.001 (7)
12	710	0.001	760	0.01 (3)
13		*	690	0.01 (3)
14	230	0.001	320	0.01 (4)
Control (pure)		*	690	0.001 (2)

Date of culturing 2/4/21—period of incubation eight to eleven days.

( ) with number indicates days necessary to kill.

* The non-fatal dose was not determined where the animals died on or after the third day; in such cases one-tenth the fatal dose rarely causes symptoms.

THE EFFECT OF SOIL AEROBES UPON TOXIN PRODUCTION

Having shown that the chemistry of the soils apparently could not account wholly for the failures in our first experiments the problem of a possible bacterial antagonism was next considered.

Soils 9, 12, 13 and 14 were cultured in glucose broth. After twenty-four hours' incubation at 37°C. each was successively subcultured several times on agar slants to eliminate the anaerobes.

Gram stains showed mixtures of hay bacilli, staphylococci and Gram negative non-sporulating rods. Each mixed culture was inoculated in a constricted tube of glucose broth along with

B. botulinus 8A, with the usual pure control. The cultures were incubated for seven days, then filtered and tested for toxicity.

The pure control filtrate killed a guinea pig (weight 220 grams) in a dose of 0.001 cc. in three days with botulism. But while some of those (weights 300 to 370 grams) inoculated with 1 cc. of the filtrates from the mixed cultures showed definite symptoms of botulism, none died.

This experiment showed conclusively that the aerobic bacteria found in these four soils were able to prevent the accumulation of strong botulism toxin in the culture, in spite of the generally accepted view that admixture of aerobes with anaerobes favors the latter through the reduction of oxygen tension.

HISTORICAL REVIEW OF AEROBE-ANAEROBE SYMBIOSIS

Shortly after Pasteur (1861) discovered his "Vibrion butyrique," the first known obligate anaerobe, he recognized that in nature this and similar organisms must often depend upon the abstraction of oxygen from their environment by aerobic microorganisms in order to grow. This explanation was accepted by Roux (1887), who utilized the hay bacillus as a technical aid in the cultivation of Vibrion septique, by Penzo (1891), who cultivated the "bacillus of malignant oedema" symbiotically with *Erythrob. prodigiosus* and *Proteus* in broth, and by Novy (1894), who did the same thing with his *B. oedematis-maligni* II (B. Novyi), adding *Bact. acidi-lactici* and a coccus to the list of successful symbionts.

In opposition to Pasteur's explanation of the symbiotic growth of anaerobes with aerobes Kedrowsky (1895) formulated a "vital ferment" theory which, however, is generally regarded as disproved by the work of Scholtz (1898), Bienstock (1899) (1903), Matzuschita (1902), Von Oettingen (1903) and others.

The symbiotic development of anaerobes other than *B. botulinus* in the presence of various aerobes has been shown in the writings of Smith (1898) (1908), Rosenthal (1902), Proca (1907), Marino (1910), Francis (1914), Torrey (1917), McCoy and Bengtson (1918), Koser and McClelland (1918), Wilson and

Steer (1918), Sturges and Rettger (1919), Rhein (1919), and Adamson (1920).

While most of these deal simply with the phenomenon of growth, Smith's (1898) (1908) observations are of particular interest in showing the inadequacy of Arnold sterilization of media intended for the production of diphtheria toxin, and those of Francis (1914) and McCoy and Bengtson (1918) in showing the lack of toxicity in tetanus cultures grown in glucose broth with certain fermentative aerobic bacteria.

Certain recent writers have used symbiotic methods technically, as for example, Koser and McClelland (1918) in securing spore suspensions of *B. tetani* and *B. putrificus*, Wilson and Steer (1918), in making fermentation tests of anaerobes; and Sturgess and Rettger (1919), in isolating *B. putrificus*.

While Bienstock (1906) found that *Bact. coli* and *Bact. lactis-aerogenes* inhibited the growth of *B. putrificus* in sugar media, Sturges and Rettger (1919) found that *Bact. coli* enhanced the putrefactive properties of *B. putrificus*.

Van Ermengem (1897), recognized the probable symbiotic rôle of sarcinae and micrococci found along with the newly discovered cause of botulism in the pickled ham under investigation. He showed experimentally that putrefactive mixtures of blood, feces, or urine failed to destroy the toxicity of cultures and that broth cultures grown symbiotically with *Erythrob. prodigiosus*, *Proteus*, *Ps. fluorescens*, and *Bact. coli* were highly toxic. The symbiotic growth in cultures of *B. botulinus* with various aerobes was also studied by Scholtz (1898), Bienstock (1903) and Proca (1907), and Dickson and Burke (1918) pointed out that *B. botulinus* is usually associated with other spores, notably of *B. subtilis*, and that "production of toxin is not prevented by the presence of other bacteria." Shippen (1919) emphasized the possible importance of symbiosis in the natural growth of this organism as an explanation of certain obscure outbreaks of forage poisoning in horses and showed experimentally that mixed cultures in broth with yeasts or *M. aureus* are still toxic. *Bact. coli* was found to inhibit toxin formation in milk, however, and we note an analogy here to the finding of Francis with

B. tetani, a sugar (lactose) not being fermented by the anaerobe but fermented with acid production by the aerobic symbiont and the cultures appearing non-toxic. Graham and his collaborators (1919) (1921) also recognized the probability of aerobic symbiosis of *B. botulinus* in nature.

There is further a tacit implication in most of the published determinations of botulinus toxin in food materials that bacterial contamination does not interfere with toxin production.

ANTAGONISTIC EFFECT OF CERTAIN AEROBIC BACTERIA UPON TOXIN PORDUCTION BY B. BOTULINUS

Having shown that mixed aerobic soil cultures prevent botulinus cultures from becoming toxic in glucose broth we then prepared to test various individual species including those recovered from the soil samples.

At this time we took account of the fact that the brain cultures of *B. botulinus* used for seeding purposes were frequently quite toxic in themselves, so that if a sufficiently large inoculum were used a culture might be rendered toxic from the beginning. We therefore determined what dilution of a brain culture would give growth in the constricted tube with 1 per cent glucose broth by making a series of dilutions, 1:10, 1:100, 1:1000, etc. Ten constricted tubes were inoculated in serial order with 1 cc. amounts of these dilutions.

The tubes inoculated with the first 8 dilutions, i.e., including the one inoculated with 0.00000001 cc., were all turbid within twenty-four hours at 37°C., and the first 5 also produced gas. In forty-eight hours all ten tubes were turbid and gas bubbles had collected below the marble seals, showing that one ten-billionth cc. brain culture is sufficient to induce growth. None of the tubes proved to be contaminated aerobically, and a filtrate of the culture inoculated with 0.00001 cc. proved toxic for a small guinea pig in a dose of 1 cc. 1:100 dilution.

Assuming a toxicity in brain cultures not exceeding 10,000 mimimum lethal dose per cubic centimeter, if only 0.001 cc. were used to inoculate 20 cc. of glucose broth, 1 cc. would contain

0.00005 cc. or less than a fatal dose for a guinea pig. Inoculations of the succeeding glucose broth cultures were therefore made with 1 cc. 1:1000 brain culture.

It was never possible in several trials to repeat these results with plain instead of glucose broth. Larger inocula are usually necessary to secure growth in plain broth.

TECHNIQUE

A mixed culture of each aerobe with *B. botulinus*, a pure culture of each aerobe, and a pure culture of *B. botulinus* comprised each test. The aerobes were mostly tested in sets of 2 to 4, so that a single pure culture of *B. botulinus* served as control for all of that particular set. Aerobic inoculations were made from agar slants just checked for purity and identity. After growth had appeared in all the tubes, each culture was spotted upon agar to determine if the aerobe grew, and if so to find if it were pure. The botulinus control was required to be free from aerobes in every case. After seven to eight days' incubation the cultures were filtered, the filtrates tested for sterility and then for toxicity on guinea pigs, as previously described.

While the tests were always made in paired sets of plain and glucose broth, only the latter failed to produce as strong toxin in the mixed cultures as in the pure controls. There was no evidence of toxin inhibition by the aerobes in plain broth, in fact the pure controls were usually less toxic than the mixed culture filtrate, though all were weak. Of course the filtrates from the pure aerobic controls were all non-toxic in a dose of 1 cc.

Lack of space prevents the presentation of detailed data of which analyses are given below—first of the results with glucose broth, and then of those with plain broth, for contrast.

RESULTS WITH GLUCOSE BROTH

Pure control filtrates fatal in doses of 0.001 cc., not in 0.0001 cc.
Mixed filtrates fatal in doses of 0.001 cc., not in 0.0001 cc.
Unidentified bacillus
B. mycoides (soil)

B. pseudotetanicus (soil)
Staphylococcus albus
B. subtilis no. 1
Mixed filtrates fatal in doses of 0.01 cc., not in 0.001 cc.
None
Mixed filtrates fatal in doses of 0.1 cc., not in 0.01 cc.
B. subtilis no. 2
Erythrob. prodigiosus
Mixed filtrates fatal in doses of 1 cc., not in 0.1 cc.
Bact. coli-communior (soil)
Bact. coli-communis
Bact. typhosum
Pure control filtrates fatal in doses of 0.01 cc., not in 0.001 cc.
Mixed filtrates fatal in doses of 0.01 cc., not in 0.001 cc.
Bact. mucosum-capsulatum
B. anthracis
Bact. alkaligenes
Mixed filtrates fatal in doses of 0.1 cc., not in 0.01 cc.
None
Mixed filtrates fatal in doses of 1 cc., not in 0.1 cc.
Bact. dysenteriae (Duval)
Bact. coli-communior (Lab.)
Mixed filtrates non-fatal in doses of 1 cc.
Bact. paratyphosum A
Bact. paratyphosum B
Bact. enteriditis
Bact. dysenteriae (Shiga)

Inspection proves that certain of the most actively fermentative members of the genus Bacterium were responsible for the less toxic filtrates of *B. botulinus*. The analogy of this result with that of Francis (1914) for *B. tetani* is emphasized.

Bact. coli-communior was the only one of the soil aerobes that was found to inhibit toxin production. At the time of isolation, which was about five months after the mixed aerobic tests were made, it was found present in soils 12 and 14. It may have been present in soils 9 and 13 at the time when the mixed soil aerobes were tested with *B. botulinus*, but may have died before an attempt was made to isolate them.

Since none of the sporulating aerobes was found to inhibit toxin production one might expect successfully to demonstrate botulinus toxin in mixed glucose broth cultures of imperfectly sterilized canned foods or other sources harboring the spores of *B. botulinus* to which sufficient heat had been applied to destroy vegetative forms before culturing. Any method selective for spores however would probably overlook certain strains of *B. botulinus*, such as our No. 8A and 78B, which do not sporulate readily and are easily killed by boiling.

RESULTS WITH PLAIN BROTH

Pure control filtrates fatal in doses of 0.01 cc., not 0.001 cc.
- Mixed filtrates fatal in doses of 0.001 cc., not in 0.0001 cc.
  - *B. mycoides* (soil)
  - *B. subtilis* no. 1
- Mixed filtrates fatal in doses of 0.01 cc., not in 0.001 cc.
  - *Bact. coli-communior* (soil)
  - Unidentified bacillus (soil)
  - *Staph. albus*
  - *B. subtilis* no. 2
- Mixed filtrates fatal in doses of 0.1 cc., not in 0.01 cc.
  - *B. pseudotetanicus* (soil)

Pure control filtrates fatal in doses of 0.1 cc., not in 0.01 cc.
- Mixed filtrates fatal in doses of 0.01 not in 0.001 cc.
  - *Bact. coli-communis* (Lab.)
- Mixed filtrates fatal in doses of 0.1 cc. not in 0.01 cc.
  - *Bact. coli-communior*
  - *Bact. typhosum*

Pure control cultures failing to grow.
- Mixed filtrates fatal in doses of 0.01 cc., not in 0.001 cc.
  - *Bact. enteritidis*
  - *Bact. alkaligenes*
  - *B. anthracis*
- Mixed filtrates fatal in doses of 0.1 cc., not in 0.01 cc.
  - None
- Mixed filtrates fatal in doses of 1 cc., not in 0.1 cc.
  - *Bact. dysenteriae* (Shiga)
  - *Bact. dysenteriae* (Duval)

THE EFFECT OF CERTAIN ACID PRODUCING SPECIES OF THE GENUS BACTERIUM ON BOTULINUS TOXIN

An experiment was made to determine if formed toxin is destroyed by the inhibitory organisms. Two lots of 400 cc. each of toxin were prepared, one in broth containing 3 per cent (an excess), the other 0.3 per cent (less than excess) glucose. Ground meat in a depth of about one-half inch was added to act as buffer. The cultures were incubated at 37°C. for four days and then filtered. That 0.3 per cent glucose was really less than excess was indicated by blackening of the meat and a terminal reaction of alkalinity. Thus, this toxin corresponded to plain broth. That 3 per cent glucose was an excess was indicated by failure of the meat to blacken and acid reaction; after filtration it still contained presumably some glucose. The two lots of toxin were distributed in tubes and tested for sterility by incubation at 37°C. over night; they were then inoculated from agar slants of the selected aerobes, and, with a control of sterile toxin, incubated at 37°C. for three days, filtered and tested for toxicity. The data are summarized herewith.

RESULTS WITH FILTRATE FROM BROTH WITH LESS THAN EXCESS GLUCOSE

Uninoculated filtrate fatal in dose of 0.1 cc., not in 0.01 cc.

Inoculated filtrates fatal in doses of 0.1 cc., not in 0.01 cc.

Bact. coli-communior (Lab.)
Bact. coli-communior (soil)
Bact. coli-communis
Bact. typhosum
Bact. dysenteriae (Duval)

Inoculated filtrates fatal in doses of 1 cc., not in 0.1 cc.

Bact. paratyphosum A
Bact. paratyphosum B
Bact. dysenteriae Shiga
Bact. enteritidis

There was really less toxin destruction by the last four species in the 0.3 per cent glucose filtrate than indicated above, for while

with a toxicity of 0.1 cc. in the uninoculated controls this amount of filtrate from these cultures failed to kill guinea pigs, it invariably produced severe symptoms, and doses of 1 cc. killed much more promptly than the control dose of 0.1 cc.

RESULTS WITH FILTRATE FROM BROTH WITH EXCESS GLUCOSE

Uninoculated filtrate fatal in doses of 0.01 cc., not in 0.001 cc.
Inoculated filtrates fatal in doses of 0.01 cc., not in 0.001 cc.
Bact. typhosum
Bact. paratyphosum A
Inoculated filtrate fatal in doses of 0.1 cc., not in 0.01 cc.
Bact. dysenteriae (Duval)
Bact. enteritidis
Inoculated filtrate fatal in doses of 1 cc., not in 0.1 cc.
Bact. coli-communior (Lab.)
Bact. coli-communior (soil)
Bact. coli-communis
Bact. paratyphosum B
Bact. dysenteriae Shiga

The most actively fermentative species were the ones that destroyed the toxin and in order to confirm this we tested the terminal hydrogen ion concentration of the following pure cultures in glucose broth after incubation at 37°C. for forty-eight hours with the following results:

	pH
Bact. enteritidis	4.2
Bact. coli-communis	4.4
Bact. coli-communior	4.4
Bact. coli-communior (soil strain)	4.4
Bact. typhosum	4.5
Bact. paratyphosum A	4.6
Bact. paratyphosum B	4.6
Bact. dysenteriae Shiga	5.4
Bact. dysenteriae Duvall	5.4
Erythrob. prodigiosus	5.6
B. mucosum capsulatum	5.6
Staphylococcus albus	5.8
B. anthracis	6.0
B. mycoides	6.0
Unidentified sporulating bacillus	6.2

B. subtilis no. 1	7.0
B. subtilis no. 2	7.2
B. alkaligenes	8.0
Broth	7.2

That acid production (pH 5.4 or less) by the aerobes was responsible for the destruction of toxin by *B. botulinus* as fast as it was formed in mixed cultures seems an inevitable conclusion. But it is well known that botulinus toxin differs from all other bacterial toxins in its tolerance for the gastric acidity, which ranges from pH 1 to pH 2. Bronfenbrenner and Schlesinger (1921, 1922) have shown that an acidity equal to that of the stomach actually increases the potency of botulinus toxin.

We performed the following experiment:

Botulinus toxin containing 1000 mimimum lethal doses per cubic centimeter was diluted 1:10 in sterile distilled water to reduce its buffer content. 0.5 cc. of this dilution was then added to 4.5 cc. of buffer solutions prepared according to the formulae of Clark and Lubs (1917) with pH values of 1, 2, 4, 5, 6, 8, and 9.4 giving a 1:100 dilution of toxin in each tube. A comparison of the buffers containing toxin with undiluted buffers by the colorimetric method showed little or no change in reaction. They were then incubated at 37°C. overnight and 1 cc. (10 mimimum lethal doses?) volumes of each injected into guinea pigs. The results were as follows:

pH	WEIGHT OF GUINEA PIG	RESULT
	grams	
1.0	150	Lived
2.0	160	Dead 18 hours
4.0	160	Dead 18 hours
5.0	180	Dead 18 hours
6.0	190	Dead 18 hours
8.0	180	Dead 6 days
9.4	160	Lived

We cannot claim from these results to have confirmed the findings of Bronfenbrenner and Schlesinger, but at any rate a pH value of 2.0 failed to destroy the toxin in the time allowed. It might be that a longer exposure comparable to what our

mixed cultures had would give a different result. The destructive action of alkali (pH 8.0 and pH 9.0) and strong acid (pH 1.0) was clearly evident.

Further experiments in line with our last one should be undertaken, for if acidities of pH 2 to 5 do not destroy botulinus toxin we shall be forced to conclude that certain fermentative aerobes destroy botulinus toxin through the operation of some mechanism involving a factor other than acid but dependent upon it.

SUMMARY

1. Toxin forming anaerobes could not be detected in soils or human feces by toxicity test of filtrates from glucose broth cultures in the constricted tube without pre-heating.

2. *B. botulinus* could not be detected by toxicity tests of filtrates from artificially inoculated soil cultures in glucose broth without pre-heating. Moderately toxic filtrates were not infrequently obtained from such cultures in plain broth, indicating a symbiotic relationship between *B. botulinus* and the bacterial flora in plain broth.

3. Meat mash medium was found to be very favorable for pure and artificially inoculated soil cultures of *B. botulinus* in both the vacuum bottle, and the constricted tube. The inhibition of aerobes in the vacuum bottle, although of some importance, is not the only factor involved in its success. The success of the meat mash medium may be attributed mainly to the high buffer content of the medium.

4. The constricted tube with marble seal was used successfully for the detection of *B. botulinus* in artificially inoculated soil cultures both in plain and in glucose meat mash medium.

5. Sterile soils did not inhibit toxin production by *B. botulinus*.

6. Impure aerobic cultures from certain soils were found to inhibit toxin production of *B. botulinus* in glucose broth.

7. Certain acid producing aerobes inhibited toxin production by *B. botulinus* in glucose broth; no inhibition occurred in plain broth.

8. Some of these acid producing organisms destroyed botulinus toxin in glucose toxin cultures.

9. Those organisms that gave non-toxic or very weakly toxic filtrates with *B. botulinus* produced the highest acidities in glucose broth.

10. A single experiment with buffer solution showed that an acidity of pH 2.0 did not injure the potency of botulinus toxin in 24 hours at 37°C.

11. The evidence points to the operation of a factor or factors other than acidity responsible for the inhibition of toxin formation or toxin destruction in mixed cultures of *B. botulinus* with acid forming aerobes.

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ON NITRIFICATION

V. THE MECHANISM OF AMMONIA OXIDATION

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INTRODUCTION

In a previous communication, on the subject of nitrification, the conclusion was reached that the oxidation of ammonia to nitrites takes place in two steps; a—one of respiration with resultant gain in energy and synchronous nitrogen absorption, b—the other of nitrogen assimilation, nitrification proper, whereby oxidation of the absorbed nitrogen takes place, the utilized portion going to make up the following cell generations, nitrous acid is split off and excreted as a non-utilizable product and energy is liberated. The phenomenon was given a diagrammatic representation.

It is the object of the present paper to study the mechanism whereby these steps take place and contribute to our knowledge of the processes of chemosynthesis occurring in Nature.

The organism used throughout these studies was an active form of Nitrosococcus isolated from Wooster soils by the methods described elsewhere (Bonazzi, 1919) and kept in Omeliansky solution for over two years without rejuvenation in soils.

GASEOUS EXCHANGES DURING NITROSOFERMENTATION

General

In 1895 Godlewsky symbolized the process of nitrosofermentation by means of equation 1.

$$NH_3 + 3O \rightarrow HNO_2 + H_2O \qquad (1)$$

This same process has been symbolized by various authors as follows:

$$2NH_3 + 2O_2 \rightarrow 2HNO_2 + 4H \quad (2)$$

$$2NH_3 + 6O \rightarrow 3H_2O + N_2O_3 + 157 \text{ cal.} \quad (3)$$

$$NH_3 \text{ acq.} + 3O \rightarrow HNO_2 + H_2O + 78.8 \text{ cal.} \quad (4)$$

$$(NH_4)_2O + 3O_2 \rightarrow N_2O_3 + 4H_2O \quad (5)$$

It is noteworthy that none of these equations is based upon a rigid experimental proof. It seems therefore that some attention should be given to the gaseous exchanges in active cultures of *Nitrosococcus*. As a matter of fact, existing data on the subject

TABLE 1

(I)	(II)	(III)	(IV)	(V)	(VI)	(VII)	(VIII)
SOURCE OF DATA	NUMBER OF CULTURE AND MEDIUM USED	NITROGEN CHANGES IN MILLIGRAM OF N			OXYGEN CONSUMED IN		$R = \frac{O}{N}$
		NH_3 transformed	N_2O_3 formed	N_2O_5 formed	Cubic centimeter	Milligram	
Schloesing, 1885A	1 soil	159.40		155.80		787.0	4.42
	2 soil	112.30		109.70		552.0	4.40
	3 soil	84.60		82.78		467.0	4.90
1885B	1 soil	208.70		198.90		1280.0	5.63
	2 soil	362.80		299.70		1886.0	5.51
Godlewsky, 1892	1 solution		22.37		78.14	111.96	4.38
	2 solution		35.86		116.25	165.10	4.03
1895*	1 solution		48.40		117.03	166.20	3.00
	2 solution		49.00		119.23	169.30	3.03

* The values given by Godlewsky in 1895 and here reproduced, were obtained by use of cultural systems in which the gaseous phase contained at the start 3.8 and 3.5 per cent of CO_2 respectively.

are extremely meagre and contradictory as may be seen at a glance from table 1 where values obtained by two observers are given for the ratio $R = \frac{O}{N}$ where O is the quantity of oxygen absorbed by the culture, expressed in gram atoms, and N is the quantity of nitrogen nitrified also expressed in gram atoms.

The discrepancies between the data presented by the two authors may well be explained on the basis of the fact that the work of Schloesing was done with soil and that in this medium

oxidations are always going on besides the ones involved in the actual process of nitrification proper. This consideration would naturally call for a greater consumption of oxygen than could be accounted for on the basis of the nitrogen nitrified. The fact also that the ratios obtained are the same for the same soil whereas they differ in the various soil types (the values of 1885a versus those of 1885b)is sufficient to point to the oxidation of some component inherent to the soil makeup.

The values obtained by Godlewsky (in solution cultures) are somewhat perplexing, but it should be here emphasized that the cultures used by this author were crude, and only in their second transfer from soil. Again, it should also be mentioned that the data were not obtained by Schloesing and by Godlewsky with the apparent purpose of studying the mechanism of ammonia oxidation. However, since they are the only values given in the literature on the gaseous exchanges in nitrification they are here tabulated to indicate that no definite concordance of existing data justifies the establishment of an equation for the phenomenon of nitrosofermentation. In this connection it should be mentioned that Meyerhoff in all his work on the respiration of the nitrous organism uses the theoretical value of $R=3$ and calculates by means of this factor the quantity of nitrogen nitrified from the quantity of oxygen consumed.

In view of the above considerations and in the hope of reaching a better understanding of the gaseous exchanges during nitrosofermentation the following series of experiments was undertaken.

Experimental

The ordinary Omeliansky solution with excess of $MgCO_3$ was used except for the introduction of ammonium carbonate in some cases in place of the sulphate. Naturally in such cases the magnesium carbonate was omitted from the solutions. The flasks with ground-glass-jointed manometers or glass flame-sealed joints, were similar in principle to those described in another publication (Bonazzi, 1921). As has been stated above, the organism used was isolated from Wooster soil.

The gaseous exchanges were studied in some cases by transferring to the special gas flasks, by means of sterile pipettes, large quantities (25 or 50 cc.) of cultures in intensive nitrosofermentation, replenishing the supply of ammonium sulphate, and sealing the flasks after obtaining samples of the gas from the system.

In other cases (cultures 331-1 and 331-2 of table 2) the sterile solution contained in the gas flasks was inoculated with a loopful of an active culture and the system closed after obtaining a sample of the gas for analysis. After varying periods of incubation, the flasks were evacuated by means of a mercury pump, the

TABLE 2

(I)	(II)	(III)	(IV)	(V)	(VI)	(VII)	(VIII)	(IX)
NUMBER OF EXPERIMENT	DAYS OF INCUBATION	OXYGEN IN AIR			NITROGEN AS NITRITE			RATIO $R = \frac{O}{N}$
		At start	At end	Lost	At start	At end	Formed	
		cc.	cc.	mg/m.	mg/m.	mg/m.	mg/m.	
271–1	14	17.14	4.06	18.68	1.68	7.47	5.79	2.82
4	14	16.79	5.16	16.62	1.68	7.17	5.49	2.71
328–1	7	16.93	6.37	15.09	9.03	13.64	4.61	2.87
7	7	16.37	4.43	17.06	9.15	14.28	5.13	2.91
6	8	16.82	5.17	16.64	8.73	13.79	5.06	2.88
3	11	17.28	4.27	18.59	9.11	14.38	5.27	3.09
4	11	17.31	1.95	21.94	8.34	14.59	6.25	3.07
2	17	16.97	5.08	16.98	9.14	14.14	5.00	2.97
331–1	50	16.34	10.52	8.31	0.00	2.66	2.66	2.73
2	22	17.10	1.61	22.13	0.16	6.79	6.62	2.92

gases collected over mercury, measured and used for analysis. In all this work ammonia and nitrites were determined by the distillation, and reduction and distillation methods developed in this laboratory (Allen and Davisson).

The results of a number of experiments are summarized in table 2, where R is again calculated as was done for table 1.

Culture 331-1 received ammonium carbonate instead of the sulphate. The average value of R is found to be 2.89 ± 0.08, ranging between 2.81 and 2.97 when the error of the various determinations is calculated according to the method of least

squares. Within the limits of error the value of R is therefore ±3 indicating that for every atom of nitrogen nitrified three atoms of oxygen are consumed by the organisms, from the surrounding atmosphere.

This conclusion conforms to the generally accepted theory, and offers justification of equations 1, 3, 4 and 5 given above.

IODINE AND NITROSOCOCCUS

General

Müntz in 1885 ascribed to the "organism qui éffectue la nitrification des matières azotées" the power of oxidizing the iodine of iodides to iodates. However, an examination of his experimental procedure shows that percolation of a nitrifiable solution, to which iodides have been added, through gravel inoculated with compost and the subsequent detection of iodic acid in the percolate is not sufficient proof to justify the conclusion that the organisms active in the oxidation of the iodides are the nitrifying organisms. Müntz's investigations were carried out long before the actual isolation of the two groups of oxidizing organisms concerned with the nitrogen cycle, so that it is not surprising if this author reached the conclusion mentioned above. Müntz explained the presence of the iodates in the Chile deposits on the assumption that the nitrifying organisms played an important rôle in their production.

From a theoretical standpoint proof of this hypothesis would be of great importance and accordingly experiments were undertaken with the object of studying the question further.

Experimental

The first step in the study was to find whether any retarding effect was exerted by iodides on the process of nitrification. Boullanger and Massoll (1903–1904) found that ammonium iodide was easily nitrified when the concentration of this salt was such that the ammonium ion was in a dilution equal to that which it attains in the ordinary Omeliansky solution.

A series of flasks, each containing 25 cc. of a culture of *Nitrosococcus* from Wooster soil in full nitrification, and which had nitrified 0.6 grams of ammonium sulphate, were used. After renewal of the ammonia supply in the form of 0.5 cc. of a 10 per cent sterile solution of ammonium sulphate, some of the cultures received 0.5 cc. of a sterile one per cent solution of potassium iodide. Tests for ammonia were made on successive dates and it was found as was to be expected from the work of Boullanger and Massoll, that no deleterious influence was exerted by the iodide upon the process of ammonia oxidation.

The next point for investigation was the actual oxidation of iodine by the organism. The same flasks used for our preliminary test received an additional dose of ammonium sulphate and after twelve more days of incubation, were removed from the incubator. Urea, and hydrochloric acid were added in equal amounts in all flasks, so as to remove the nitrites by the Piccini reaction (1879), and allowed to stand in the cold for several days to complete the removal of the nitrites. Then, after the addition of barium hydrate solution, they were filtered and the residues washed with cold water several times to remove the traces of nitrites that might be found, due to incomplete reaction. After digesting the residue in weak HCl the mixture was again filtered and the filtrate tested for the presence of iodic acid by the addition of a 0.25 per cent solution of sodium sulphite using starch as indicator. The blue color would in this case only develop in presence of iodates. Tests with four different strains were concordant in showing iodides but no iodates present at the end of such an experiment.

Apparently no appreciable, if any, oxidation of the iodine could be observed in the cultures and it seems likely that in the soil another group of organisms (possibly the nitrate former) is responsible for the phenomenon detected by Müntz. It should be kept in mind however in this connection that Omeliansky (1902) could not prove for this latter group of organisms the presence of a function of oxidation on the sulphurous and phosphorous acids.

IRON AND NITROSOCOCCUS

General

Winogradsky and Omeliansky (1899) found that iron is necessary for the process of nitrification and Kaserer (1911) attempted to find a condition whereby iron could be made more available for the bacterial cells by dispersion as an aluminium-silico-phosphate. In a previous communication from this laboratory (Bonazzi, 1919) it was found, however, that the iron aluminium-silico-phosphate of Kaserer, when added to nitrifying solutions proved of no avail for the hastening of the oxidative action of *Nitrosococcus*.

Remy and Rösing (1911) and later Allen (1919) in a study of the conditions of iron in cultures of *Azotobacter* showed that the presence of colloidal iron was extremely beneficial to the process of nitrogen fixation by this organism. The work of Allen opens up an interesting field in the physiology of bacteria and points to the possible function of iron as an oxygen carrier and as a protective colloid for the precipitated phosphates.

In the present study however iron is considered from another standpoint, i.e., its behavior in the cellular metabolism. In the nitrifying solutions iron is introduced as a ferrous salt. In view of the fact that iron has been suggested as being involved in processes of oxidation (Fenton and collaborators 1894, 1899 and 1900) by acting as a carrier of oxygen for other substances, while itself undergoing oxidation, it was thought that a study of the changes undergone by this element in a nitrifying solution might throw some light on the process of ammonia oxidation.

Experimental

Cultures 344–0, 344–2, 344–3, 344–6, and 344–7 were used. All except 344–0, which was an uninoculated control, were young cultures and in active nitrification when used. Two cubic centimeters of each culture were placed in tubes and acidified with 5 drops of HCl: each tube was then placed in an apparatus from which all the oxygen was removed by means of alkaline pyrogallol. By means of a dropping funnel fitted in the appara-

tus each tube received a few drops of $FeK_4(CN)_6$ with the results given in table 3. Under these conditions the ferrocyanide yields a blue precipitate of potassium-ferri-ferrocyanide only in presence of ferric salts. At the same time some of the acidified culture received in the laboratory air a few drops of $FeK_3(CN)_6$ with the results given in the second line of the table.

When old cultures are tested which have oxidized all their ammonia, and have not received a new addition of this compound for some time, no reaction for ferrous iron is obtained.

The blue precipitate obtained with active cultures is sometimes slow to appear and on close observation appears to be constituted of deeply colored blue cellular clumps. The clear unacidified or acidified filtrate from even a very active culture fails to show the presence of either ferric or ferrous iron. Cells washed free of nitrites on Chamberland filters became deeply blue on prolonged contact with $K_3Fe(CN)_6$ in acid solution.

TABLE 3

TREATMENT WITH	334-0	334-2	334-3	334-6	334-7
$FeK_4(CN)_6$	Deep blue precipitate	Very light blue tinge in all inoculated			
$FeK_3(CN)_6$	No color	Dark blue color in all inoculated			

It is thus evident that the iron in the uninoculated solutions undergoes a very profound oxidation whereas in the inoculated it is partly retained in the ferrous form. In addition to this, it should be mentioned that when cultures were started in an Omeliansky solution which had been prepared for so long as to have all its iron transformed to the ferric form, tests of the control uninoculated solutions failed to give a reaction for ferrous iron, whereas the active cultures yielded a decidedly blue cellular mass.

THE PROCESS OF OXIDATION

General

It is not necessary here to review all the theories that have been advanced for the explanation of the phenomena of oxidation since an interesting discussion of this subject may be found in Mellor's book (1909) and Kastle's monograph (1910).

In previous communications from this laboratory it was shown that the presence of a KOH container in a cultural system stops nitrification, and this fact may be correlated with the greater concentration of the free NH_4 ion in the culture solution, resulting in a greater toxicity to the organism. This also stands out from the work of Meyerhoff (1917). To this should be added the action of the absence of all traces of CO_2 in the atmosphere and medium.

Aside from the fact that Omeliansky (1902) failed to isolate an oxidase from the cells of the nitroso organism nothing is known with respect to the intimate mechanism of this phase of assimilation. It is true, Chodat (1906) suggested the existence of a nitroxidase but he really brings forth nothing in the line of experimental proof of his suggestion when he states that since ammonium carbonate is in reality an ammonium carbamate, the nitroxidase is in truth a carbamase, capable of attacking only this type of linking.

In these processes of oxidation the organism must take an active part by means of an intracellular mechanism since various attempts made in this laboratory failed to demonstrate an extracellular nitroxidase thus corroborating the findings of Omeliansky. An oxidase may be considered as a joint system of peroxide-peroxidase; in the presence of a peroxide, oxygen is liberated by the peroxidase and used in the oxidation of oxidizable substances. The peroxide need not be peroxide of hydrogen, and, as is clearly pointed out by Bach and Chodat, may be an organic peroxide. If a mechanism were found whereby the oxidation of ammonia could be brought about by means of stored energy a step would be taken towards an understanding of the mechanism of the biological oxidation of nitrogen in Nature.

In order to study this question, several attempts have been made in the direction of bringing about an oxidation of ammonia to nitrous acid, in the cold, by chemical means. Weith and Weber (1874) found that ". . . . hydrogen peroxide and ammonia reacting upon each other gave rise to nitrous acid. . . . ." These authors claimed thereby to have found the origin of nitrous acid in Nature.

Carius (1874) found that ozone and ammonia gave rise to ammonium nitrite as follows

$$(NH_3)_2 + (O_3)_4 \rightarrow (NH_4)NO_2 + H_2O_2 + (O_2)_4$$

and that

$$(NH_4)NO_2 + H_2O_2 \rightarrow (NH_4)NO_3 + H_2O$$

Thus the fact was established that hydrogen peroxide is formed as a transitory product in the reaction, capable of carrying the oxidation one step further. These findings were corroborated by Hoppe-Seyler (1883), Riegler (1897) and McIntosh (1902). However Hoppe-Seyler (l. c.) as well as Griess himself, when recommending his reagent for nitrites (1880), and again Wurster (1886) cautioned against the fact that the presence of hydrogen peroxide interferes with the color development of the naphthylamine and sulphanilic acid reagent for the detection of nitrites.

Fausto Sestini (1904) as well as Russell and Smith (1906) found that a limited oxidation of ammonia could take place in presence of ferric hydrate in an atmosphere saturated with ammonia, and Leoncini and Pieri (1909–1914) found nitric acid to be formed by the action of boiling solutions of manganese dioxid upon urea, cyanamide and dicyandiamide. This last compound gave rise to small quantities of nitric acid even at 30°C.

The conclusions, that these various authors reached, were to the effect that although nitrites and nitrates might appear, the quantities are not important from a practical standpoint. This is true when these actions are considered as taking place independently in the soil, but it is the opinion of the present author that this phase of their importance is secondary. What these phenomena really do mean, is that the active oxygen is liberated from compounds that contain it, and becomes active in the oxidation of ammonia and some organic compounds.

A mechanism whereby peroxides are continually formed and degraded with liberation of the energy stored during the peroxidation, is an oxidase system: a mechanism whereby this peroxide oxygen is used in the oxidation of ammonia becomes extremely

important in the present connection. Experiments were accordingly undertaken to ascertain whether, according to the earlier investigators, nitrites are formed in the presence of peroxides in the cold.

Experimental

To avoid the difficulty of detecting nitrites in presence of hydrogen peroxide the latter compound was precipitated in the form of barium peroxide and the filtered supernatant liquid used for the necessary tests by means of the Ilosway-Lunge modification of the Griess reagent.[1]

TABLE 4

NUMBER OF FLASK	HOURS OF INCUBATION	CUBIC CENTIMETERS OF SOLUTION USED IN EACH FLASK				RESULT: NITRITE REACTION
		$(NH_4)_2SO_4$	$MgCO_3$	Na_2CO_3	H_2O_2	
1	0	25	2		5	Negative
2	24	25	2		5	Intense
3	48	25	2		5	Intense
4	0	25		1	5	Negative
5	24	25		1	5	Intense
6	48	25		1	5	Intense

For the experiments on oxidation described in the accompanying paragraphs, 25 cc. portions of a 0.2 per cent solution of $(NH_4)_2SO_4$, in nitrite-free water, were used together with 1 cc. of a 5 per cent solution of Na_2CO_3 or 2 cc. of a suspension of $MgCO_3$. Ground-glass-stoppered flasks were used throughout. The peroxide used was a 3 per cent (Parke, Davis) solution and of this 5 cc. were used in each case. The arrangement of the experiments and some of the results obtained are summarized in table 4. The temperature of incubation was 27°C.

Nitrites are thus actually formed under the conditions of experiment. The proof once obtained for this fact, as also of the purity of the hydrogen peroxide used, many other trials were

[1] It was thus found that a 3 per cent solution of H_2O_2 could be made, by means of this procedure, to give a negative test with the ether chromate method for the detection of peroxides. According to Mellor (1912) this test is sensitive to a concentration of one part of H_2O_2 in 80,000 parts of water.

carried out and could here be described. However it is sufficient to state that repeated trials showed that whereas nitrites were formed from the sulphate of ammonia in Omeliansky solution with $MgCO_3$, they failed to appear when the carbonate of ammonia was used in absence of a base. Also it was found that a solution of ammonium sulphate in water would not nitrify in absence of a base, whereas the formation of nitrites from a pure solution of NH_4OH and of $(NH_4)_2CO_3$ could take place when in absence of magnesium carbonate. The necessity of an alkaline reaction might also explain the failure encountered when $(NH_4)_2CO_3$ was used in Omeliansky solution without magnesium carbonate or other base. The peroxide used was in a weakly acid solution.

This formation of nitrites was found to take place also in an atmosphere free of CO_2 although at a rate slower than in ordinary air. Nevertheless even under these conditions of CO_2 removal ammonium carbonate in Omeliansky solution failed to nitrify.

As has been stated, oxidation will take place even in solutions of the pure salts, without addition of other materials, and this is an additional proof that the chemical process is independent of any other constituent of the Omeliansky solution. Experiments with this solution from which the various salts had been omitted one at a time, served to prove this assertion. In every case nitrite was formed, after 24 hours of incubation, except in those cases where $MgCO_3$ or $(NH_4)_2SO_4$ had been omitted.

It would seem reasonable to assume that, if the mechanism of biological oxidation of ammonia is tied up with the action of a peroxide, it should be possible to demonstrate the presence of one of these compounds in the active cultures of the organsim of nitrosofermentation. The fact that the ether-chromate test for peroxides fails to give any results with active cultures, might open the possibility that the peroxide concerned in the biological nitrification is an organic peroxide other than the peroxide of hydrogen. However, the transitory nature of a peroxide in an active culture, or any other oxidizing system, should be here emphasized on theoretical grounds.

Again, an active culture filtered and washed free of nitrites with water, or freed of its nitrites by Piccini's reaction, fails to indicate the presence of an oxidase or even a peroxidase when tested with the guaiac gum solution. Omeliansky relates a similar experience. Another line of attack should then be devised.

Were a peroxide active in the biological odixation of ammonia, it should be possible to find the mechanism whereby the peroxide oxygen is liberated. Accordingly some experiments were undertaken with the object of establishing whether cultures in active nitrosofermentation are capable of breaking down the H_2O_2 molecule with liberation of oxygen.

For this purpose a small shaking machine was used which imparted a uniform motion to two flasks; one flask containing the living culture the other the control solution. The control solution was in some cases freshly prepared or incubated sterile Omeliansky solution, in other cases a culture in active nitrification which had been pasteurized previous to use. In every case 2 cc. of culture, living or variously treated, were intimately mixed in the flasks with 10 cc. of a 1.5 per cent solution of hydrogen peroxide, and the oxygen developed was collected over water at constant room temperature and measured in graduated burettes at definite intervals.

The experiments carried out, all gave similar results and only a few are given in table 5 as typical examples. The values given in this table are also graphically reproduced in figure 1.

From the results just given it is possible to derive the statement that the bacterial cells in a culture of *Nitrosococcus*, or some of their by-products are capable of bringing about the breakdown of hydrogen peroxide.

By filtering an active culture, washing it with acetone and finally drying it with ether, a white powder is obtained which is principally made up of precipitated magnesium carbonate. When this powder is used, after suspension in water, and compared with another portion of the same material which has been kept at boiling water temperature for ten minutes in a water bath, values are obtained which are given in table 6.

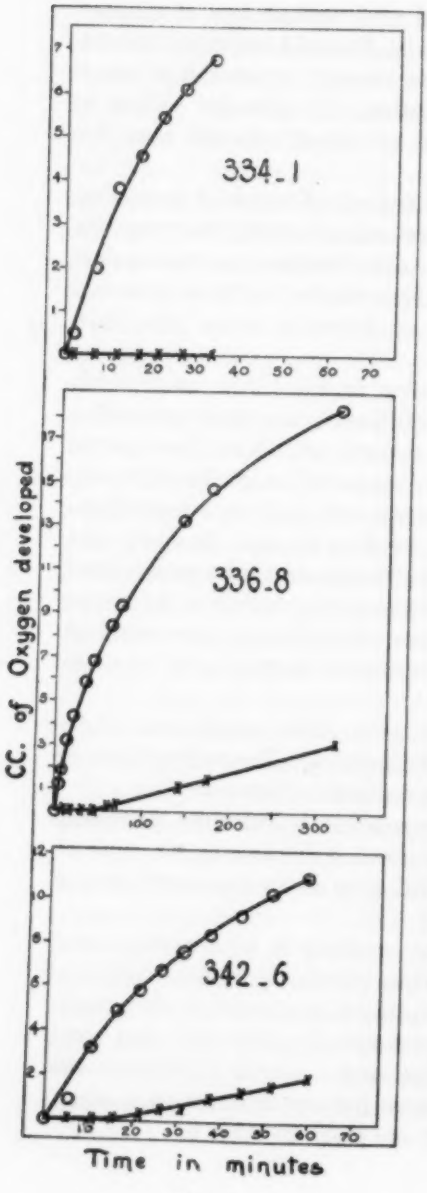
334.1
336.8
342.6
CC. of Oxygen developed
Time in minutes

FIG. 1.

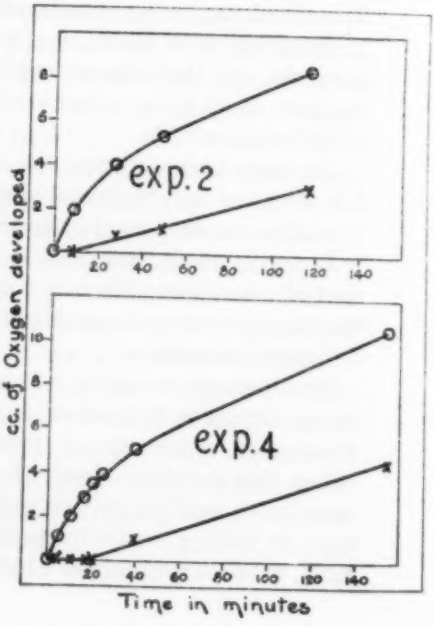
exp. 2
exp. 4
cc. of Oxygen developed
Time in minutes

FIG. 2.

The results given in table 6 are graphically shown in figure 2. The acetone-ether preparations used in these experiments had stood for three days in the dry condition. However, when a culture which had nearly finished dissolving its $MgCO_3$ precipitate, due to the formation of large quantities of nitrous acid,

TABLE 5

CULTURE 334-1			CULTURE 336-8			CULTURE 342-6		
Time in minutes	Oxygen developed in cubic centimeter		Time in minutes	Oxygen developed in cubic centimeter		Time in minutes	Oxygen developed in cubic centimeter	
	Culture	Control		Culture	Control		Culture	Control
0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
2	0.50	0.00	6	1.90	0.00	5	0.95	0.00
7	2.00	0.00	13	3.25	0.00	10	3.25	0.00
12	3.85	0.00	19	4.35	0.00	16	4.95	0.00
17	4.60	0.00	33	5.95	0.00	21	5.85	0.25
22	5.50	0.00	41	6.90	0.00	26	6.75	0.45
27	6.15	0.00	61	8.50	0.30	31	7.65	0.45
34	6.85	0.00	71	9.40	0.40	37	8.45	0.95
			143	13.35	1.25	44	9.30	1.30
			175	14.80	1.60	51	10.17	1.45
			326	18.47	3.25	60	11.16	1.85

TABLE 6

EXPERIMENT 2			EXPERIMENT 4		
Time in minutes	Oxygen developed in cubic centimeter		Time in minutes	Oxygen developed in cubic centimeter	
	Culture material	Boiled control		Culture material	Boiled control
0	0.00	0.00	0	0.00	0.00
8	1.82	0.00	5	1.10	0.00
28	4.00	0.80	11	2.10	0.00
50	5.30	1.17	17	2.85	0.00
118	8.27	2.95	21	3.50	0.17
			25	3.95	0.20
			40	5.10	0.90
			155	10.60	4.55

was centrifuged, washed several times with water, acetone and then ether, poured into a crystallizing dish to dry and subsequently experimented upon, it failed to give any appreciable liberation of oxygen from the peroxide due no doubt to the weak but distinctly noticeable acidity of the reacting system.

Thus the mechanism of peroxide breakdown is truly "catalytic;" it is destroyed by heating and is completely lacking in fresh Omeliansky solution as well as in this same solution to which nitrites have been artificially added. The curves obtained when this solution is used sterile, approach very nearly a linear function and are indicative of the surface action of the system. In the cultures instead, there is a strong activity at first with a subsequent retardation as time goes on, the curve tending to become parallel to the abscissa.

DISCUSSION AND CONCLUSIONS

As has been mentioned earlier in this contribution, peroxide oxygen could not be demonstrated in the cultures even with as delicate a test as the ether-chromate reaction.

Nevertheless a mechanism is undoubtedly developed whereby this compound is broken down with the liberation of free oxygen (a peroxidase reaction) and this leads to the conclusion that this compound or compounds of similar nature play an active rôle in the process of nitrosofermentation. Unfortunately experiments to investigate the action of hydrogen peroxide additions to active cultures have so far given no indications of value, but this is possibly due to the excessive concentration of this compound in the immediate vicinity of the cells.

Iron is found in active cultures partly in the ferrous state and this leads to a special train of reasoning.

When ferrous sulphate is acted upon by an alkali (NaOH) in the absence of air, a white precipitate is formed of ferrous hydroxide which as long as it is kept free of oxygen remains white, but becomes yellow, green and later dark-brown to black in the presence of the smallest traces of oxygen indicating that this compound has changed in structure and has acquired the structure of ferrous oxide-peroxide. This latter compound is far more active both chemically and electrically than the white ferrous hydroxide, and, as was shown by Quartaroli (1917) and by Baudisch (1921) is concerned with many of the changes in the oxygen content of compounds which, alone, are quite stable in ordinary conditions.

The return of the highly oxidized form of iron to the compound of lesser oxidation, by yielding its oxygen to the bacterial cells would release the energy gained in the first step of oxidation and thus facilitate the inception of the second step, of nitrification proper, which as has been seen, can easily be reproduced by artificial means. By attacking the ferrous-oxide-peroxide the bacterial cells allow nitrification to proceed maintaining the ratio of $\frac{O}{N}$ equal to that found in the present work ($\pm$ 3).

The iron acting as a carrier of oxygen would be far more active in a shallow layer in continual motion than in a deep layer, thereby hastening nitrification; this has been found to happen in previous work in this laboratory. In this connection it should be mentioned that Ashby (1907) found nitrification taking place very actively in presence of "ferric hydrate" and "iron rust" as the only bases present. The meaning of these results is evident in the light of the knowledge gained in the present investigation. The iron in Ashby's cultures not only acted in virtue of its weak alkalinity, as was argued by this author, but also as an active oxygen carrier. Here the results obtained by Russell and Smith, reported above, are extremely interesting.

The breakdown of the peroxide with the liberation of the "activated" oxygen is accomplished by a mechanism inherent to the cell itself, similar to that exhibited by the cells when in presence of the high concentrations of hydrogen peroxide used in the present study.

Accepting Bach and Chodat's conception of oxidase action, we can conclude that the iron by its mechanism of autooxidation fulfills in the nitrifying cultures the functions of the peroxide, while the cells furnish the mechanism for the liberation of the oxygen thus bound.

A direct proof of the existence of an oxidase by the guaiac test can not be obtained due to the presence in the solution of other compounds (biproducts of the bacterial activity itself) which will themselves oxidise the guaiac gum. Nevertheless it appears probable that the system may be represented by a sequence such as follows.

$$Fe'' + O \rightleftarrows Fe''' \cdot O \therefore Fe''' \cdot O + \text{peroxidase} + NH_3 \rightleftarrows HNO_2 + \text{peroxidase} + Fe''$$

The ferrous-hydroxide-peroxide plays here the rôle which the peroxide plays in the system of Bach and Chodat.

To this fact may be added the observation made often in the course of these studies that the iron cannot be washed out of the cells even by the action of strong nitric or hydrochloric acids.

The change that nitrogen undergoes in passing from the ammoniacal to the nitrous form is one which involves the valency of the element. From a scientific standpoint therefore, as well as from a practical, the process of biological oxidation of ammonia presents great interest. Lafar (1911) in discussing this question states; ". . . . as is apparent from this equation, their power (nitrobacteria) differs from that of the nitrosobacteria, inasmuch as the latter convert the pentavalent nitrogen of ammonia into the trivalent nitrogen of nitrous acid, whilst the nitrobacteria reconvert the element into the pentad condition. . . . ."

The fact that the two types of organisms are capable of such widely different functions should be confronted with their similarity of behavior in cultural conditions, a thing that at present finds no explanation.[2] The question of the autotrophy of the organisms concerned is of importance in this connection and should be correlated with the powers which these two organisms have of utilizing, by means of their cellular complex, the energy changes of the element nitrogen.

Naturally the change in the condition of the nitrogen is subordinate to the mechanism of oxygen activation, since the cultural system itself free of bacteria is inert, from the standpoint of nitrous and nitric acid formation; it should be realized that the

[2] It is true that according to Chodat the nitrosoferment oxidizes only ammonium carbonate because this compound is really an ammonium carbamate with nitrogen in the trivalent state. The hypothetical nitroxidase of Chodat would only be capable of oxidizing the amino group of this compound, by an action similar to that of the enzymes of the laccase type which oxidize commercial cyanamide; the hydrogen peroxide and carbon here acting as secondary catalysts. However if this were the case, it is difficult to find an explanation for the complete disappearance of the ammonia from a nitrifying system, a fact that indicates that the total molecule of the carbonate has been utilized. Besides the existence of a nitroxidase has not as yet been proven.

simple acceptance of the living bacterial cells as catalysts in the ordinary meaning of the word will not explain the changes that occur during nitrification, unless a study is made of the conditions that allow the bacterial cells to produce and utilize a mechanism for the activation of the inactive atmospheric oxygen. It is the object of this paper to show that the mechanism whereby this phenomenon, preliminary to the process of nitrification proper, and intimately connected with it, takes place is one in which iron is active by virtue of its property of activating the inert oxygen of the air; the mechanism appears to be intracellular. This explains why Omeliansky and the present author failed to isolate a nitroxidase from active cultures of *Nitrosococcus*.

From the practical standpoint the present study finds an application in the new interpretation offered for the process of nitrite and nitrate formation in Nature. The question of autotrophy of the organisms, active in these changes, has for a long time been a question-mark in the study of bacteriology. Various authors have also attempted to show that the autotrophy is only a condition forced by artificial cultural environment. Still no other group of organisms has yet been found which exhibits so marked a plasticity of protoplasmic functions as to become in cultures completely and hereditarily unrecognizable, from a physiological standpoint, from their normal selves.

Undoubtedly, although some authors are trying to make of the group of nitrifying bacteria an aberrant group, highly specialized and derived from the common heterotrophic forms, we ought to consider them instead as very primitive forms, protobacterial so to speak, which retain as yet their autotrophic functions and are active geological agents in the breakdown of the slowly soluble rocks.

The facts often observed relating to the beneficial effects to be derived from the additions of organic matters to impure cultures of the organisms of nitrification, as well as the benefits to be derived from the application of organic fertilizers in agricultural practice, should not be explained by assuming a direct action of these substances upon the organisms themselves, a theory disproved by the work of Winogradsky and Omeliansky

and in fact the only work of the kind ever carried out by means of absolutely pure cultures;[3] but by the indirect effect of the contaminating forms on the medium in which nitrification takes place, i.e., the solution of mineral substances which prove necessary to the intimate mechanism of nitrification proper. The findings of Müntz relating to an abundant nitrifying flora in the peaks of alpine mountains are sufficient proof that the above considerations are not far from the truth.[4]

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[3] In this connection, a recent note by Winogradsky in Comp. Rendus., **175**: 301, 1922 will be found very appropriate.

[4] The author wishes to express his thanks to Dr. E. R. Allen, formerly of this laboratory, for his helpful criticisms during the progress of this study.

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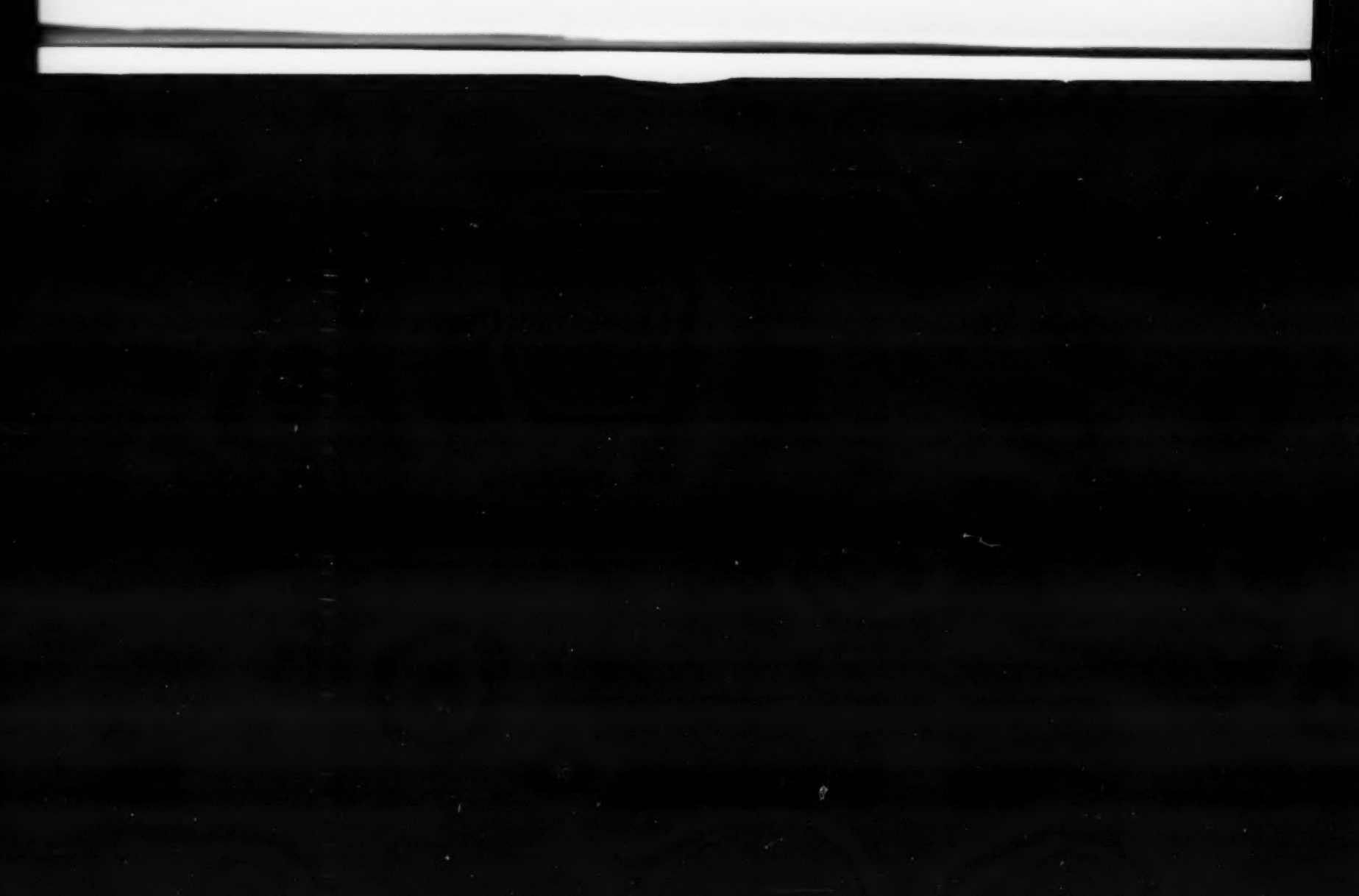

FURTHER STUDIES ON THE MORPHOLOGY OF BACTERIA

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Official bacteriology, if I may venture to use the expression, still regards the morphology of bacteria as very simple. The cells of a certain species are supposed to be fairly uniform and to multiply, only by fission. As a matter of fact the very system of classification of bacteria is based on this theory. Many investigations have shown, however, that the theory needs revision. A thorough review of the literature concerning this problem is to be found in the excellent work by F. Löhnis[1] recently issued.

Besides reviewing the literature Löhnis discusses a theory of his own, according to which bacteria develop cyclic changes called life-cycles, including an organized and an amorphous stage. During the latter stage the cell-walls disintegrate and the protoplasm of different cells mixes. From this unstainable "symplasm" new individual cells develop. If these claims could be proved to be true, it would, I think, be a most interesting discovery, throwing a new light on the problem of variation of bacteria. As is well known, studies of the Twort-d'Herelle phenomenon have shown the great importance of the variation of bacteria. It has been found that not only the lytic agent but the ageing of the cultures induces variation. How does this happen? What rôle do the metabolic products play and how do the hereditary factors work? All these problems are very difficult to approach before we know whether there is any exchange of protoplasm between the cells of the bacteria.

In studying the morphology of *Coryneb. diphtheriae* I have been very anxious to find the structures figured by Löhnis

[1] Memoirs of the National Academy of Sciences, vol. xvi, 1921.

especially since Almquist, an author of my own country, describes the same things under the name of "plasmodia." He claims that very small and numerous organized individual cells develop from the "plasmodia." I am able to confirm the statement that the "plasmodia" or "symplasm" are easily seen for example with *Coryneb. diphtheriae*, in cultures of some age; but I cannot abandon the old view that they are merely slimy agglomerations of cells or, in other words, a "zoogloea." My reasons are that the masses always contain some cells, perhaps empty-looking which, however, are possibly able to grow, and that the production of slime, sometimes in gorgeous masses, in ageing cultures is very common with bacteria. It may be true that the cells growing from the "plasmodia" are sometimes very numerous; but this can be explained in another way, as we shall see later.

Löhnis also describes one mode of interaction between the plasmatic substances of the bacterial cells called "conjunction." This process consists of a "direct union of two or more individual cells" exactly comparable with the copulation of yeasts. The well-known V-forms of *B. subtilis* and *Coryneb. diphtheriae* are the result of such a conjunction, the cells being connected "by bridges or by beaks." With regard to the *Coryneb. diphtheriae* I have followed the development of these bridges and have found that they are not secondary conjunctions, but residues left when the new cell forms from the old one just like the threads connecting the cells of a yeast mycelium.

Nevertheless, though I cannot adduce any evidence in support of Löhnis' discoveries, I am very much inclined to believe in them, since it is improbable that bacteria form an exception to the rule that a species dies at length without sexual rejuvenescence.

Beside the above-mentioned processes Löhnis describes several kinds of asexual multiplication common to all bacteria. One of these is the formation of gonidia. In analogy with the large trichobacteria, as for example Cladothrix and Leptothrix, bacteria form within the cells small bodies which are able to grow, either while still in the mother cell, or when liberated by the bursting of the cell wall. If the cells containing gonidia have

changed their shape into giant forms they are called gonidangia. The club form of the Actinomyces is regarded as such a gonidangium. "Very many of the so-called involution forms of spherical, pearshape, and clubshape are, in fact, gonidangia." I am convinced, however, that many of the pictures explained by Löhnis as growing gonidia are only budding and branching vegetative cells. He says that the great multitude of forms seen with *Bact. coli* and *Bact. typhi* and especially in the pictures of these bacteria published by Hort are due to the development of gonidia before they are liberated from the mother cell-wall. As I have pointed out in numerous earlier papers, these "fantastic" forms are just the same as occur in yeast and must be regarded as normal vegetative cells. A proof that Löhnis exaggerates the importance of the gonidia is to be found on page 144 of his monograph, where he says that "it still remains to be studied whether the eosinophilic granules, which are present in blood-cells, are actually cell-products or bacterial gonidia."

On the other hand, it cannot be denied that fishing from old bacterial cultures, containing no intact cells, or very few, but including masses of easily stainable small granules, often gives an abundance of colonies on the agar plates. It is then very tempting to explain this growth, as many have done, as originating from the granules. But to supply a proof is exceedingly difficult, and I doubt whether it is possible to provide convincing pictures. In diphtheroids and other bacteria I have often seen the organisms in the form of a pure culture of filaments, which in growing old have become more and more granulated, and at last have broken up into granulated masses. Plating from such a culture has, as has been mentioned above, given numerous short bacillary forms. I have supposed that the granules are contracted masses of protoplasm separated by empty spaces in the ageing filament and not definite organized gonidia, since they differ widely in size and shape. The name I have found most adequate is "fragmentation spores" a term used by Bostroem in Actinomyces. These formations are, I think, the cause of the striped appearance of *Coryneb. diphtheriae*. They are not to be confused with the polar granules, being real granulas and lying in vacuoles, where they exhibit a dancing movement.

Furthermore Löhnis tells us about some other bodies serving the purpose of multiplication which he calls regenerative bodies. They are of different nature and are produced either by the gonidia or by the symplasm and they are "generally characterized by their being easily and deeply stained by aqueous dyes, by their different appearance as compared with the vegetative cells (globular, oval, or irregular), by their ability to reproduce normal cells immediately or after having propagated as such by fission or by budding, by their distinctly increased resistance against drying, heat, or other detrimental influences." Beside the "regenerative bodies" he describes "arthro-spores" and "microcysts," the latter being formed by "transformation of a whole vegetative cell into one, usually relatively large, resting body" the former "by the segmentation of the vegetative cell and the transformation of these segments into fairly resistant reproductive organs." The thickening of the wall is characteristic of both, but usually the arthro-spores are more cubical and the microcysts more rounded in shape. The microcysts can grow to vegetative cells or change into gonidangia or divide into several equal parts, each able to give rise to a new cell. The author says that "what we now call regenerative bodies is a collection of different reproductive organs, whose proper separation and classification must be left for the time when more data shall be available."

In studying bacteria I have always followed as a guide the theory that bacteria are closely connected with the fungi proper. This theory at once throws light on the pleomorphism of the bacteria and also on many problems of variation. According to this theory, it is natural that the bacteria cells should differ widely in shape and that new variants of more or less constancy, should rise in a culture. In the same way I have tried to explain the structure of the bacterial cell and finally also some peculiar bodies which I have seen in the case of *Coryneb. diphtheriae*, *Bact. coli* and *Bact. typhi*. These bodies evidently belong to one of the above mentioned groups of Löhnis. For my part, however, I should prefer another name to those used by Löhnis since these bodies are most similar I think to the so-called chlamydospores of the fungi. What then is a chlamydospore? This name is

given to a peculiar kind of resting form having thick walls and being a little more resistant than common vegetative cells. Many of the Eumycetes, for example Favus, show mycelial filaments with circumscript swellings or bulges. Such swellings are often transformed into chlamydospores by the flowing of the protoplasm from other parts of the filament to the swelling, which afterwards surrounds itself with a thick wall and becomes granulated. Owing to the abundance of protoplasm and the presence of the thick membrane, the body takes stain in a higher degree than the other cells. The remaining parts of the filament soon die and the spore is free. The "resting cells" of yeast show a very great resemblance to these chlamydospores. The only difference is that here the whole cell is transformed into a resting form. Chlamydospores according to Plaut are demonstrated on the text-figure below. Now it may be mentioned, first of all, that many bacteria show just the same swellings, as is well known to every one familiar with ageing bacterial cultures. Sometimes the swellings are situated in the end of the filament, which then assumes a club-like shape, common to many bacteria, for example, *Mycob. tuberculosis* and *Coryneb. diphtheriae*. In other cases the swelling has a peculiar triangular form. The formations just described are mostly to be seen on pictures of "involution forms;" but many writers have accepted them as organs of multiplication. It may be remembered that Plaut utters a warning against the confusion of these swellings and spores and other higher formations found in fungi. How much more easily does such a confusion take place when we are dealing with bacteria which are of so small a size.[2]

FIG. 1. CHLAMYDOSPORES ACCORDING TO PLAUT

Sometimes, however, you will see the whole development of chlamydospores from these swellings even in the case of bacteria. *Bact. typhi* shows these forms, especially when grown at low temperature and on drying agar as Almquist has pointed out, and

[2] In Kolle-Wasserman, Handl. der path. Mikroorganismen, Bd. v, Jena, 1913.

Coryneb. diphtheriae shows the same forms when left for a week or more on Löffler serum. Figures 8, 12 and 16 demonstrate such bodies from a culture of *Bact. typhi*. As to *Coryneb. diphtheriae* I refer to an earlier article. Neither of these bacteria, however, is very suitable for studies of these formations, since they appear rather irregularly and only in ageing cultures. I have found excellent material in a Corynebacterium originally isolated by Rosenow from a case of Hodgkins disease and kindly provided me by R. R. Mellon. The corpuscles appear in the cultures of this bacterium in great abundance even after twenty-four hours' growth on plain agar as is to be seen on figures 1, 7 and 13. A scrutiny of the living microörganism under high magnification showed that the filaments produced swellings which, just like those of Favus, were situated everywhere, even in the ends, which then looked like the "clubs" of *Coryneb. diphtheriae*. Thickening of the cell-wall and gathering of the protoplasm inside was clearly seen in many of the swellings. On the other hand, such bodies were also seen free, with only faint traces of the filament attached to them. These traces and bodies on the ends of branched filaments prove that the development is that hinted at above and not the reverse, as many have imagined. As a matter of fact the pictures obtained from the development of the chlamydospores in a hypha is sometimes strikingly similar to those of the outgrowth of a spore to a filament, especially when dealing, not with long filaments, but with rods or bacillary forms and when the body is placed at the end. But the observation of bodies in both the ends of the rod immediately solves the question. As far as I can see, Meirowsky[3] has made such a mistake in interpreting his pictures of *Mycob. tuberculosis*.

But those bodies, are they not gonidangia, asci, or whatever you like to call them? With the very same microörganism Mellon[4] has found bodies containing small motile corpuscles getting free by the bursting of the wall and giving rise to new individuals. I have not been able to cultivate these bodies; and

[3] Studien über Fortplanzung von Bacterien, Spirillen und Spirochäten, Berlin, 1914.

[4] Journ. Med. Research, 1920, **62**, 61.

they are not the same as those described above. The chlamydospores, as I like to call them, never contain any formations indicating a multiplication, and they grow by budding just as yeast does. The evidence of budding was easy to obtain, since the bodies put in new broth and removed from this in a high dilution of thionin showed beautiful "budding discs" just like those of yeast and other microörganisms described by me in an earlier paper.

Finally I want to emphasize the fact that I do not deny the existence of other more complicated forms in bacteria, but merely report what I have seen and have not seen. In vain I have tried to find gonidangia or asci. Even with *V. cholerae*, where large round, thick-walled granulated cells very suspiciously similar to the organs of multiplication, are common in ageing cultures, I have never found anything of that kind. On figures 2, 4 and 5 some of these bodies of *V. cholerae* are demonstrated. It is, as will be seen, big enough to be easily studied.

SUMMARY

The author discusses several theories concerning peculiar forms in bacteria supposed to be organs of sexual and asexual multiplication. He also describes some spherical, ovoid or club-shaped bodies found by himself with *Bact. typhi*, *V. cholerae*, *Coryneb. diphtheriae* and other species, which he regards as chlamydospores.

EXPLANATION OF THE PLATE

Figures 3, 6, 9, 14 and 15 are taken from wet preparations obtained by transferring the microörganisms from the culture to a drop of the staining fluid, which was then covered with a glass. In such a thin layer of fluid the microörganisms lie motionless at least for a time long enough to allow photographing. The other pictures are taken from preparations fixed by drying the slides in the usual way. The staining fluid was in all preparations a highly diluted solution of thionin in water.

Figures 1, 7 and 13 (*Corynebacterium lymphogranulomatis* Rosenow). Slanted agar culture twenty-four hours old. Many chlamydospores in different stages of development.

Figures 3, 9 and 15. The same bacterium with a chlamydospore developing near or in the end of a hypha. In the two latter the microörganisms have become more or less club-like.

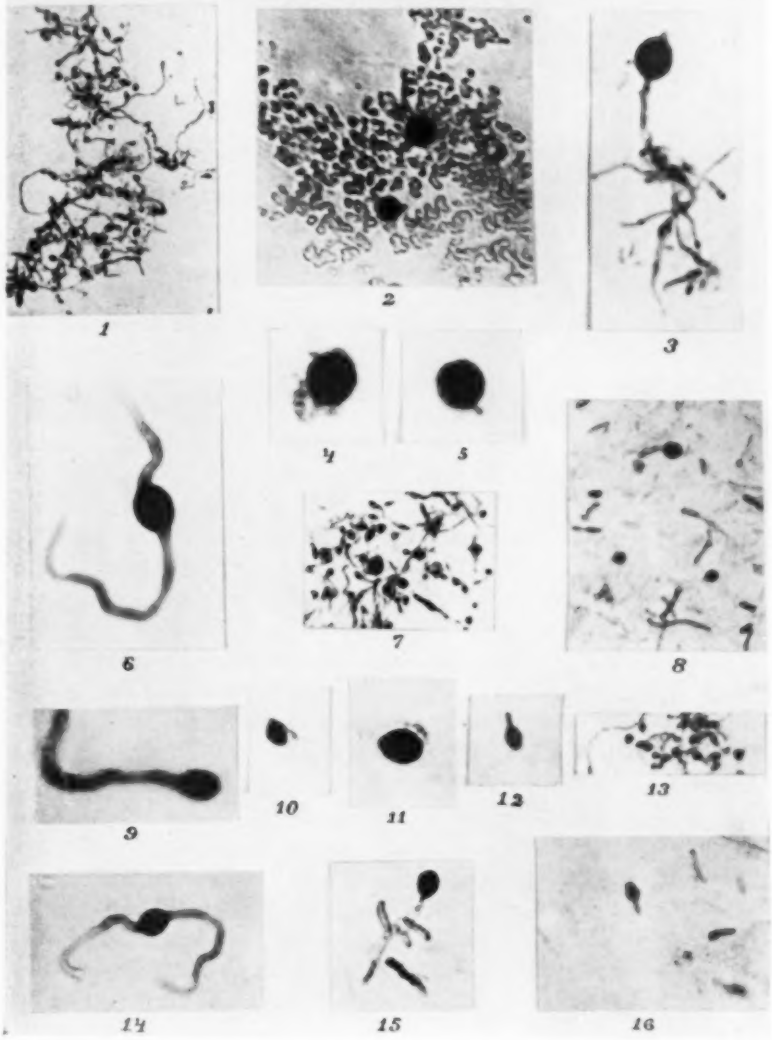
Figures 6 and 14. Development of the spore in the middle of the hypha. The swelling of the thread, the thickening of the wall, and the gathering of the protoplasm to the spore are clearly seen.

Figures 10 and 11. Free spores, the former still with remnants of the thread adherent to the wall, as is mostly the case.

Figures 2, 4 and 5. Round bodies in a culture of *V. cholerae* on slanted agar three weeks old. In figure 2 the size of the bodies can be compared with that of the usual form of the microörganism. In figure 5 the body shows a definite granulation. The bodies are supposed to be resting cells.

Figures 8, 12 and 16. Pictures from a slanted agar culture of *Bact. typhi* three weeks old. In the two latter a chlamydospore is seen to develop at the end of a rod, giving to this a club-like shape. The next stage, with the rod shrinking away and the spore more or less liberated, can be observed on figure 8.

Plate I



(Bergstrand: The Morphology of Bacteria.)

CLOSTRIDIUM PUTRIFICUM

II. MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL STUDY

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Interest in this peculiar but extremely important organism has increased within the past few years. Since Sturges and Rettger (1919) brought out again the outstanding features of this anaerobe, among them that of delayed development in pure culture, investigators have expressed widely different views. An example of the uncertainty concerning the entity of the organism in question is given by the British Medical Research Committee. In their first report on the classification and study of the anaerobes found in war wounds (Special Report Series, No. 12, 1917) certain cultural reactions attributed to it are given rather fully and there is every reason to assume that the committee at that time believed in the existence of a definite species which they were calling *Bacillus putrificus*. Later (1919) in their Special Report Series, No. 39., they do not make reference to it as a distinct species, but infer that "many of the cultures of *B. putrificus*, so-called, are really mixtures of *B. cochlearius* or *B. tertius* with *B. sporogenes*." They make no effort to list the cultural characters of the organism, and simply offer a short review of the work of previous investigators, which does not identify it in any way as a distinct species. The Lister Institute (London) does not list cultures of *C. putrificum* in its National Collection of Type Cultures (1922) and the writers were unable to procure from them any cultures of this organism for comparative studies.

Hall has, in a way, shown equal uncertainty in regard to the identity of *C. putrificum*. In the differential key to the sporulat-

ing obligate anaerobes given in Jordan's General Bacteriology, this key being taken from some of Hall's unpublished data, Jordan puts *C. putrificum* in a group separate from *C. cochlearius*, The basis of differentiation is a very fundamental one, that of liquefaction of coagulated albumen and blackening of brain. Following close upon the publication of this classification scheme, however, Hall (1922) specifically states that his studies show that *C. putrificum* and *C. cochlearius* are identical. Hall, in the latter work at least, was evidently working with pure *C. putrificum*, but the descriptions are incomplete and in some respects erroneous. This is especially true regarding growth in milk, in which he reports no visible action. The description of *C. cochlearius* given by the British investigators is very much at variance with that suggested by Hall, the Research Committee claiming it to be identical with *C. putrificum*. The writers made a preliminary examination of the type strain of *C. cochlearius* received from the Lister Institute and found it to correspond to the description given in the British Report No. 39, and to be very unlike *C. putrificum*.

It must be admitted that many of the investigators working with this anaerobe have made use of impure cultures, and often have not had the real *C. putrificum* in the cultures described. The writers make no original claims as to the use of pure culture: they are interested here merely in defending the specific entity of this anaerobe, and wish to offer complete proof to substantiate their point.

The bacillus of Bienstock is an anaerobe which has constant characters and which has been described a number of times in undoubted pure culture, but each time quite incompletely. Since Bienstock's original cultures were impure, his first descriptions (Bienstock, 1894, 1899, and 1901) are worthless, except in one particular: it is certain, from his descriptions, that he had reference to an anaerobe having round, terminal spores, and that this mixture of organisms caused putrefactive decomposition of meat. Tissier and Martelly (1902) were evidently working with pure cultures, for they emphasize the inability of *C. putrificum* to attack sugars, even glucose, except very slightly

and with the production of such small amounts of acid that the medium never becomes distinctly acid. This characteristic was confirmed by Rodella (1905) and also by Bienstock himself a year later (Bienstock, 1906). Distaso (1911) specifically brings out the characters of *C. putrificum* as given by Bienstock in 1906 and designates his various strains as belonging to the tetanus group, the round, terminal-spore group. Distaso, however, described two species, one of which is the true *C. putrificum*, while the other resembles in some respects both *C. putrificum* and "*B. paraputrificum*" (Bienstock). His *B. putrificus-filamentosus* has the specific properties claimed by Bienstock, and approaches in almost every detail the strains isolated by Sturges. His description, however, is incomplete, and is not sufficient to establish the specificity of the organism.

On the other hand, a very large number of workers have described cultures which are undoubtedly entirely different from the type strain of Bienstock. Among these may be mentioned Metchnikoff (1908), Aschoff (1917), Medical Research Committee (1917 and 1919), Kendall, Day and Walker (1922) and Kahn (1922). The misunderstanding concerning this much-buffeted organism has been due in a large measure to the meagre descriptions given in the literature. Hall's work (Hall, 1922) not only is incomplete, but there are some errors which would certainly complicate identification if allowed to stand in the present form. The writers (1921) in a preliminary paper, attempted to point out the true characters of *C. putrificum* and later (1922) published such of their data as would establish the specific entity of the organism. The need for a complete tabulation of the morphological and cultural characters is yet felt. In the present work these are given detailed consideration. Biochemical studies of the action of the organism on peptone and glucose are included to support the qualitative cultural observations. Strains of *C. putrificum* isolated by Sturges were used in this study.

For the sake of clearness, the exact technique employed will be given. This feature is often neglected and the reader has too little information upon which to make comparative studies.

Only ordinary laboratory media were used and the simplest technique employed. The composition of the media and the methods used will be given under each separate heading, or references cited which furnish the particular information.

MORPHOLOGY

The morphology of *C. putrificum* may be said to be characteristic. At least it differs very considerably from the other common anaerobes, with the possible exception of Bienstock's "*B. paraputrificus.*" It is a long, slender rod, quite often curved, and in old cultures frequently occurs in long filaments. It is much more slender than the other anaerobes, being generally from 0.5 to 0.7μ broad and from 7.0 to 8.0μ long. There are often shorter forms, but these are not the common type. The spore is round and strictly terminal, and very large in proportion to the rod (See Reddish and Rettger, 1922, plate I, fig. 1). In egg-meat medium (see Rettger, 1906), in which the organism grows and preserves its viability particularly well, and in which our observations as to morphology were made most frequently, spores are not to be seen until about the tenth day or after. It may require two or three weeks for the typical spores to appear in appreciable numbers in this medium. The appearance of spores accompanies the beginning of apparent digestion of the meat. The spores remain in the rods for several days after they are first observed. At this stage they are present in large numbers. Hall (1922) apparently failed to note numerous spores in any of his cultures. This may have been due to his using a less favorable medium or not allowing sufficient time to elapse before the examinations were made. No spores have been observed by us on glucose agar and in glucose broth, although no persistent efforts were made to demonstrate their presence in these media.

C. putrificum is weakly Gram-positive, even in young cultures, and may be said to be on the border line between Gram-positive and Gram-negative. It is almost uniformly Gram-negative in old cultures. It stains well with the ordinary aniline dyes. In studying the staining property, various kinds of media were used,

but cultures in egg-meat medium have proved in our hands to be most reliable for this purpose.

Relatively young egg-meat cultures are actively motile, and motility is not completely lost during sporulation.

COLONY FORM

Surface colonies on glucose agar (1 per cent glucose, 1.6 per cent agar, 1 per cent Difco peptone, and 0.5 per cent Liebig's beef extract)[1] even after prolonged incubation are small, delicate and almost transparent. The edge is irregularly, but not deeply dented; there are no outgrowths and no apparant fringe. The structure appears to be somewhat granular, but not coarse. (See Reddish and Rettger, 1922, fig. 2). The aid of a hand lens is necessary in order to see the colonies distinctly, since they are by far the most delicate formed by any of the anaerobes studied by the writers. At least three days are required for the colonies to show any appreciable development.

Depth colonies in glucose agar tubes (1 per cent glucose in 1 per cent nutrient agar) do not appear until the third of fourth day, and then they are mostly in the extreme lower portion of the tube. For the study of deep colonies, inoculation was made at the surface of the medium with a pipet, using a plain broth culture of the organism, the mixing being accomplished by rolling the tube between the hands; the tubes were incubated aerobically. Apparently the conditions in deep agar tubes favor rapid development better than surface cultivation. The development of colonies in the deeper portion of the medium only signifies the strict anaerobic conditions required. The nucleus of the depth colony is dense, and there are fine, but not long, hair-like radiations from the central portion. Because of the indistinctness of the depth colonies, sharp observations could not be made.

[1] Plates poured with a thick layer of this medium were allowed to dry, inverted, at 37°C. over night and then streaked with a bent glass rod from a saline suspension of an egg-meat culture of the organism. Incubation was at 37°C. for three and for ten days under strictly anaerobic conditions. The time allowed for incubation before removal for description was ten days.

CULTURAL CHARACTERS

Egg-meat. Freshly sterilized egg-meat medium was cooled quickly after sterilization, inoculated immediately from ten to fourteen day egg-meat cultures of the organism, and incubated aerobically at 37°C. There was little change, aside from a slight turbidity, until a week to ten days after inoculation, depending upon the amount of inoculum used. By this time there is usually slight softening of the solid matter and the meat and egg particles become finer. This disintegration is at first most apparent in the upper portion of the medium, but latter progresses into the lower layers. Reddening of the meat has by this time become very pronounced. By the end of the second week there is some decrease in the bulk of the solid matter and digestion is very apparent; this pronounced change is accompanied by the evolution of the foul odors that are characteristic of real putrefaction. Because of the evaporation of the liquid resulting from the long incubation, there is never a very deep layer of liquid over the undigested residue, such as is noted in egg-meat cultures of *C. sporogenes*. The meat is darkened only slightly, the deep red color being dominant throughout the digestion. Only when the meat has become entirely decomposed is there definite browning; the residue in old dried cultures is, however, almost black, but this change does not take place until after several months. There is no apparent gas production at any time. The digestion of the egg-meat is never rapid, and the evaporation of the supernatant liquid almost keeps pace with it.

Milk. Deep tubes of freshly sterilized skim milk were cooled quickly in water and then inoculated with one loopful of an egg-meat culture of the organism,[2] after which sterile melted paraffin was added to form a seal about half an inch in thickness. The tubes were incubated at 34°C, and not 37°C. because the paraffin softened at the latter temperature. Under these conditions there is slow precipitation of the casein and later almost complete

[2] A loopful of a ten to fourteen day meat culture is as a rule large, for the solid particles cling to the loop in the form of lumps of partly digested meat, in which much enzyme is carried.

digestion. The time required for this action varies with the inoculum, but more definitely with the age of the culture from which inoculation was made. This is illustrated by the following experiment. Four freshly sterilized deep tubes of milk were inoculated with a large loopful of six-weeks old egg-meat cultures of four strains of *C. putrificum*. Four other deep tubes of freshly sterilized milk were inoculated with a large loop of twenty-four-hour egg-meat cultures of these same strains. All eight tubes were sealed with sterile melted paraffin and incubated at 34°C. The four tubes which were inoculated with the old (digested) cultures showed slight digestion at the end of twenty-four hours, and practically complete digestion of the casein within four days. On the other hand, the tubes inoculated with the young cultures did not show any change until the twelfth to fifteenth day, when there was some digestion of the slightly precipitated casein. One of the tubes did not show any signs of growth for four weeks, after which time there was slow digestion of the casein. In all of the last four tubes the digestion took place slowly. The rapidity of the action in the first set was probably due to the preformed enzymes carried over.[3]

Broth. Ordinary plain nutrient broth made with beef extract was inoculated with a large loop of a three to four weeks old egg-meat culture and covered with sterile melted paraffin. Tubes without paraffin were placed for comparison in an anaerobic jar and both sets were incubated at 34°C. The growth after two days was slight to fair. In glucose broth the growth is little if any better. The turbidity is never sufficiently great to cause at most more than a very slight sediment.

Gelatin. When gelatin is inoculated with a three to four weeks old culture, rapid liquefaction takes place (one to five days), but when young cultures are used as inoculum the liquefaction is correspondingly slow, and may require from two to three weeks. Liquefaction progresses at about the same rate in plain and glucose gelatin.[4]

[3] This may account for the varying results claimed with this medium. Some investigators report rapid digestion of casein, while others claim that there is no apparent action at all in milk.

[4] Deep, narrow tubes incubated aerobically.

Loeffler's serum. Ordinary Loeffler's blood serum used for growing *Coryneb. diphtheriae* was employed in place of the inspissated horse serum recommended by certain English investigators. Growth was observed after three days' incubation at 37°C. in an anaerobic jar, following heavy inoculation with old cultures. Digestion did not take place completely until after five days.

BIOCHEMICAL REACTIONS

Fermentation tests. The following substances were used in testing for acid and gas:

Pentoses: xylose and arabinose.
Hexoses: glucose, levulose, galactose and mannose.
Disaccharides: lactose, sucrose, maltose and trehalose.
Trisaccharides: raffinose and melezitose.
Polysaccharides: soluble starch, dextrin, glycogen and inulin.
Methyl pentose: rhamnose.
Glucosides: salicin, amygdalin, esculin and inosite.
Alcohols: mannitol, glycerol, erythritol, dulcitol and sorbitol.

These were used in plain nutrient 1 per cent agar, adjusted to pH 7.0 before sterilization. The test substances were added to make a 0.5 per cent solution and the medium filled into tubes which had been previously sterilized, thus allowing for a shorter sterilization period. The tubes were sterilized at 15 pounds for ten minutes, cooled quickly to 45°C. and inoculated as soon as possible. Inoculations were made with a 1 to 1 suspension of a twenty-four hour plain broth culture of the organism, 0.5 cc. being added at the surface of the medium. The tubes were then rolled vigorously between the hands to insure thorough distribution of the organisms, and incubated aerobically at 37°C. A control plain agar tube was also included. None of the test substances were apparently fermented. At least there was no visible gas production and tests for acid by the brom-thymol-blue method were negative. In testing for acid the indicator was added to the melted culture after a week's incubation. Colonies were easily visible by the third or fourth day in all of

these media. As is shown below, glucose is probably feebly attacked, but not enough to yield acid in sufficient amount to show in the brom-thymol-blue test as used above.

Glucose-consuming power. One per cent glucose broth was used for determining quantitatively the amount of glucose consumed by *C. putrificum*. The broth was filled into large tubes, 20 cc. per tube, sterilized and cooled quickly before inoculation with 0.5 cc. of a twenty-four hour plain broth culture.[5] Six tubes were so inoculated and quickly placed in six separate anaerobic jars. In this way it was possible to open one tube at a time without disturbing the anaerobic conditions under which the others were kept. Quantitative determinations of glucose were made by the method of Benedict (see Cole's Practical Physiological Chemistry, 1919, p. 127). Readings were made at the end of 12 hours, 36 hours, 60 hours, 7 days and 14 days. The hydrogen ion concentration of the cultures was also determined at the same time.

Glucose is attacked but slightly, and not enough to be considered as indicative of definite saccharolytic property on the part of the organism. A loss of only 0.1 per cent glucose is recorded (See Reddish and Rettger, 1922, chart 1). Allowing for experimental error, the amount consumed may be even less. At least, the ability of the organism to consume this sugar is not an outstanding or important feature of its metabolism. The hydrogen ion changes did not keep pace with the glucose reduction, due to the simultaneous production of alkali. The pH dropped from 7.3 to 6.6, but the degree of acidity is not such as would be readily measurable in melted agar cultures by any of the tests now employed, as noted above. The amount of chemically determined glucose was reduced from 0.727 to 0.625 per cent in fourteen days at 37°C. *C. sporogenes*, on the other hand, reduced the glucose from 0.727 to 0.385 per cent in fourteen days. *C. tetani* consumed more dextrose than did *C. putrificum*, reducing it from 0.727 to 0.541 per cent in fourteen days. Therefore, relatively speaking, at least, *C. putrificum*

[5] The use of a twenty-four hour seed culture was made possible by making a heavy inoculum in the original plain broth.

is in a class by itself so far as its action on glucose is concerned. For all practical purposes, it may be considered non-saccharolytic.

Peptolytic property. For determining the degree of peptolytic action on commercial peptone, ordinary plain broth, in 35 cc. amounts in large tubes, was inoculated with 1 cc. of a twenty-four hour plain broth culture. The inoculation was made immediately after sterilization and cooling, and the tubes were placed in separate jars and put under anaerobic conditions, the number of tubes and jars being sufficient to allow for determinations at the end of 12 hours, 36 hours, 60 hours, 7 days and 14 days, without disturbing the unused tubes. The following tests for following the destruction of the peptone were used in the present studies: (1) quantitative biuret, (2) Sörensen, (3) ammonia, and (4) amino-acids. The biuret test is essentially that employed by Vernon (1903). A modified Sörensen test,[6] as used by Kendall and Walker (1915) and others was used for determining approximately the ammonia and amino-acids present. The use of the ammonia test has been widely employed both in this country and abroad. The test as used in this work makes use of the iodine-thiosulphate titration (see Sutton's Volumetric Analysis, 8th edition, 1900). Van Slyke's modification of Folin's test was employed for liberating the ammonia in the broth cultures, the gas being collected in acid solution of known strength. The iodine-thiosulphate method of titrating was used because of its greater accuracy. The amino-acids were determined by the micro-method of Van Slyke (1913–1914, 1915).

The albumoses peptone, and higher polypeptids are attacked very little before the third day, but by the seventh day there is considerable change. There is, by this time, a great decrease in the biuret-giving substances, with a consequent increase in the ammonia and Sörensen figures. After one week's incubation the ammonia and Sörensen figures increase only slightly over the seven day reading and there is practically no further destruc-

[6] In the Sörensen test 5 cc. of neutral formaldehyde were employed and the titration with $\frac{N}{20}$ NaOH made immediately after the formaldehyde was stirred into the solution.

tion of the biuret-giving substances. The delayed action on peptone is not surprising, because of the general tendencies of the organism toward poor development in pure culture in the early stages of growth. Since peptone is more easily attacked than the more complex protein of meat, the changes noted as taking place in seven days are comparable to those taking place in meat after a longer period. Another index of the destructive activity of the organism and its enzymes on egg-meat and proteins in general is the almost complete conversion of the amino-nitrogen into ammonia-nitrogen in the experiments. (see Chart 2, in the previous paper, 1922).

Pathogenicity. A 1 to 10 suspension of a two weeks old egg-meat culture when injected intraperitoneally causes no changes whatever in the condition of the animals tested (white mice and guinea pigs).

Although the media and methods used in this study have been very simple, sufficient data are given to point out clearly the essential features of this peculiar organism. The characteristics as brought out here coincide with certain outstanding features ascribed to it by Bienstock. It is hoped that in the present work, the characteristics exhibited and described may be of material use in placing the organism in its proper group in the classification of anaerobes, and also offer a more or less complete method of identification with the kinds of media most used in this country. A definite effort has been made to employ only such media as can be procured and made easily.

According to the work here presented, *C. putrificum* is not only a definite and distinct species, but should be placed in a group of its own among the known anaerobes. It is proteolytic, peptolytic and at most only very slightly saccharolytic.

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THE TITRIMETRIC ADJUSTMENT OF THE HYDROGEN ION CONCENTRATION OF BACTERIOLOGIC CULTURE MEDIA

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Adjustment of the reaction of bacteriologic culture media to a definite hydrogen ion concentration has seemed such a difficult procedure that some laboratories have not even yet given up the inaccurate and obsolete method of adjustment by titration which was standard a few years ago. Yet that method was fundamentally sound if only an indicator whose range included the desired end point had been used.

Some bacteriologists have tried, and it is a notable tendency among students to attempt, to translate the nomenclature of the older method into that of the newer one, but this is possible only with unbuffered acids and alkalis or in solutions of known and unvarying buffer content, and is manifestly impossible for the organic mixtures in use for cultivating bacteria. Even "standard" formulas are likely to involve important variations in the chemical composition of their ingredients that affect the hydrogen ion concentration of media made with different lots.

Bacteriologists seem generally also to have assumed, incorrectly, that adjustment of a culture medium to a definite pH value necessarily involves the predetermination of the hydrogen ion concentration of the medium, and as a result adjustment by the colorimetric method has generally been based upon what is

actually titration, but titration in tubes[1] with a comparator block rather than in the more convenient evaporating dish or beaker.

It is the purpose of this note to emphasize (*a*) that a culture medium may be adjusted to a definite hydrogen ion concentration without actually determining its initial pH value, (*b*) that the amount of acid or alkali required to adjust may be titrated by a procedure almost identical with the old standard method, using beakers or evaporating dishes, and (*c*) that meticulously standardized (i.e., normal) acid and alkali are not required.

Clark has repeatedly, and particularly in his recent book,[2] mentioned that adjustment of the reaction of culture mediums is essentially a matter of titration, but McCrudden[3] has given what seems to me the most cogent statement of procedure yet published, as follows:

> To adjust media to any desired hydrogen ion concentration, $\frac{N}{10}$ alkali is added drop by drop to 5 cc. of the somewhat diluted media containing indicator until, as shown by comparison with the standards, the desired hydrogen ion concentration is reached. From the amount of alkali required for 5 cc. the amount needed for the whole batch of media can then be calculated. Sterilization of the media shifts the pH about 0.2 towards the acid side. Allowance should be made for this.

But McCrudden advocates the comparator, which, after all, is considerably more complicated in its use than a pair of evaporating dishes or beakers.

[1] Hurwitz, Meyer and Ostenberg, On a colorimetric method of adjusting bacteriological culture media to any optimum hydrogen ion concentration, Proc. Soc. Exp. Biol. and Med., 1915, **13**, 24.

Clark and Lubs, The colorimetric determination of hydrogen ions and its application in bacteriology, Jour. Bact., 1917, **2**, 1, 109, 191.

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Foster and Randall, A study of the variations in hydrogen ion concentration of broth media, Jour. Bact., 1921, **6**, 143.

[2] Clark, The Determination of Hydrogen Ions, Williams and Wilkins Co., Baltimore, Maryland, 1920.

[3] McCrudden, The determination of hydrogen ion concentration, Pub. Health Reports, 1922, **37**, 334.

We in this laboratory have for some time been using and teaching a method of titrimetric adjustment of hydrogen ion concentration of culture media that is really simpler than the method of adjustment to a particular titratable normality.

For general purposes we assume that a pH value of 7.0 approximates the optimum reaction. The same principles of adjustment apply, of course, to any other pH value, using appropriate indicators.

To adjust a medium of unknown reaction to pH 7.0, determine first whether it reacts as an acid or as a base to brom-thymol-blue by adding 0.3 cc. of a 0.04 per cent solution of this dye in 50 per cent alcohol to 10 cc. in a white evaporating dish or a beaker against a white background. A yellow color indicates an acid medium, a blue color indicates an alkaline medium, and a green color indicates a neutral medium. Highly colored (brown or yellow) alkaline media may also appear green and should be diluted so that the normal color of the medium does not interfere with that of the dye.

If the medium is acid, ascertain the number of cubic centimeters of weak alkali required to match the color of the medium with that of an equal amount of a standard buffer solution carefully adjusted to pH 7.0 and containing the same amount of indicator in a similar vessel.

If considerable dilution of the medium and indicator occurs either in obliterating the original color of the medium or in titration, it should be compensated in the standard by an equivalent addition of distilled water.

It is not necessary that the alkali used in titration or in adjustment be standardized in terms of normality; but the relative strength of titrating fluid and adjusting fluid must be known. It is generally convenient to use approximately N/1 NaOH for adjustment and an exact dilution of 1:100 for titration; the same number of cubic centimeters of the titrating solution required to neutralize 10 cc. of medium should be used of adjusting solution to neutralize each liter. A check test should be made after the adjustment is complete, and again after sterilization. The final hydrogen ion concentration of the finished medium should be recorded.

No general rules can be laid down as yet regarding the correct allowance to make for changes in reaction during sterilization but any careful technician by repeated observation of the changes that occur in the different types of medium ought soon to establish a set of working rules to guide procedure in his own laboratory.

Certain observations upon the titrimetric method of adjusting hydrogen ion concentration are of interest.

The standard buffer solution must, of course, be made up in the first instance with extreme care. Those that I worked with were prepared by Foster and Randall[1] and were carefully checked by the electrometric method. Such buffer solutions are apparently little changed by ageing in good glass bottles; even the paraffine lining of the bottles which often parts from the glass during warm weather and allows free contact between solution and bottle is probably superflous. But I wish particularly to emphasize that subsequent lots of a given buffer solution that is almost exhausted may be made simply and easily by the titrimetric method, using the residue of the lot to be replaced for the color standard, without the tedious recrystallization of salts and redistillation of water required for the initial standard.

Furthermore, in the use of the buffer solution as a color standard, our ordinary distilled water, which reacts distinctly acid to brom-thymol-blue, had no apparent effect upon the green color of our pH 7.0 buffer even on dilution of 1 part of buffer to 20 parts of water; at 1:40 however there was a slight difference in the direction of acidity. It is apparent therefore that considerable economy of standard buffers may be effected by such dilution.

The question of the proper temperature for titration received answer in experiments involving undiluted and diluted (up to 1:20) buffer (pH 7.0) similarly tinted with brom-thymol-blue. Boiling indicates a slightly higher alkalinity, presumably through driving out CO_2. But since the green color returns similarly on cooling either to the temperature of incubation (37°C.) or to room temperature, titration without boiling is advocated as a routine practice.

The amount of dye to use may be varied within rather wide limits according to the desire of the titrator for while the *quantity* or intensity of color depends directly upon the amount used, the

quality of the indicator color of brom-thymol-blue is essentially the same when used in quantities of from 0.1 to 1 cc. of 0.04 per cent alcoholic solution in 10 cc. of our standard buffer (pH 7.0) diluted or undiluted. It is of course essential to have the same concentration of dye in the medium or solution being titrated and in the standard.

I have paid particular attention to the feasibility of diluting the medium for the purpose of obviating color interference. If results obtained with an almost colorless medium (meat infusion broth with 2 per cent peptone and 0.5 per cent NaCl) can be considered as applicable to one with more color, it seems, firstly, that the hydrogen ion concentration reading is unaffected by dilution of 1 part medium with 10 parts distilled water, notwithstanding that the water alone showed a markedly yellow color (see table 1) at room temperature and a strikingly blue color on boiling. This statement certainly holds also for mediums ranging in pH value from 6 to 8.

TABLE 1

*The effect of dilution of medium upon the hydrogen ion concentration reading**

	COLOR COLD	COLOR ON BOILING
10 cc. medium undiluted	Pale green	Slightly yellow
3 cc. medium plus 7 cc. distilled water	Pale green	Slightly yellow
1 cc. medium plus 9 cc. distilled water	Pale green	Slightly yellow
10 cc. distilled water undiluted	Yellow	Blue

* Each mixture contained also 0.3 cc. 0.04 per cent alcoholic solution of brom-thymol-blue.

Clark and Lubs[1] have shown both mathematically and experimentally that considerable dilution of a buffered medium is permissible before the hydrogen ion concentration is visibly altered.

On the other hand, several experiments have shown that the amount of alkali calculated as required to adjust media that were too acid, to a pH value of 7.0 is greater when such calculations are based upon the titration of samples diluted too far. In other words, a comparison of separate lots of a medium ad-

justed upon the basis of titration with varying dilutions of the medium always showed that those adjusted by titration of too highly diluted samples were made too alkaline. The differing quantities of alkali so calculated in one such experiment are shown in table 2.

In these experiments it was always observed that as the buffer in the medium was decreased by dilution the effect of each drop of titrating fluid became more marked, i.e., the indicator became

TABLE 2

*Effect of dilution of media on amount of alkali required to adjust to pH 7.0**

	OBSERVED N/100 NaOH REQUIRED TO MATCH STANDARD AT pH 7.0	FACTOR	CALCULATED N/1 NaOH REQUIRED TO ADJUST 1 LITER	COLOR OF ADJUSTED MEDIUM 100 cc.
	cc.		*cc.*	
10 cc. medium undiluted	9.0	1	9.0	Green
5 cc. medium plus 5 cc. distilled water	4.4	2	8.8	Green
3.3 cc. medium plus 6.6 cc. distilled water	3.2	3	9.6	Bluish green
2.5 cc. medium plus 7.5 cc. distilled water	3.0	4	12.0	Blue
2 cc. medium plus 8.0 cc. distilled water	2.3	5	11.5	Blue
1 cc. medium plus 9 cc. distilled water	1.2	10	12.0	Blue

* Each mixture contained also 0.3 cc. 0.04 per cent alcoholic solution of bromthymol-blue.

more sensitive to equal increments of titrating fluid. Since exactly similar results were obtained with boiled and unboiled distilled diluting water, and the acidity of the water was insufficient of itself to account for the discrepancies, it is not quite clear why titration of diluted media should overshoot the mark but it is evident that too great dilution in titration should be avoided.

AROMA-PRODUCING MICROÖRGANISMS

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Cultures of bacteria often produce specific odors of various degrees of intensity and thus enable us to recognize readily several species. Frequently the odor is unpleasantly noticeable in places where mass cultures of bacteria are kept.

The odor produced by the cultures may be a general group character or an individual specific characteristic. The typical putrefactive bacteria can for example, be recognized by their specific odor, while cultures of yeasts, molds and actinomycetes are readily recognized by their peculiar aroma. Old cultures of *Erythrob. prodigiosus* produce a typical odor of trimethylamine, *B. fitzianus*, the odor of sweat (caproic acid ester), *B. odorificus*, the odor of rotted manure, etc. The same is true in respect to pathogenic bacteria: old cultures of *Mycob. tuberculosis* produce a specific odor, *Ps. pyocyanea*, a sweetish odor, resembling that of the lime-tree flowers, *M. tetragenes* and *M. pyogenes*—an odor partly resembling that of glue, etc.

Large numbers of similar examples can be cited, but these do not interest us at present. There is a special group of microorganisms, the representatives of which can be properly named "aroma-producing microörganisms," including those species which produce in culture a strong ethereal aroma of varying intensity, reminding one of the odor of fruits: strawberry, pineapple, apple, pear, muskmelon, etc. Here belong also those microörganisms, which give the "characteristic odor" to various products used for food and beverage, such as milk, cheeses, butter, wine, etc. The names of the species belonging to this group are correspondingly formed by the addition of epithets: *aromati-*

cus, aromafaciens, odorus, odoratus, esterifaciens, fragi, fragariae, nobilis, etc., and often different authors describe different species under the same name. There are in the literature three different *Bact. aromaticum*—those of Beijerinck, Pammel and van der Leck, and three *Bact. odoratum*—of Henrici, Burri and Weiss.

Aroma-producing microörganisms are quite abundant in nature; they are found in the soil, water, hay, in various plant infusions, in milk, etc. Several species have been isolated from manure and human excreta, in one case, that of cholera (*Bact. praepollens* Maaszen). The accumulation of organic acids and alcohols in the culture, mutually combining *in statu nascendi* and forming complex esters, is the cause of formation of the complex ester odor. The esters of acetic, butyric and valerianic acids are those most often produced.

The property of forming pleasant aromatic products represents a changeable character, which is more or less rapidly lost on artificial cultivation. To regain the lost characteristic, the culture is transferred upon the corresponding natural substratum: bacteria forming a strawberry aroma,—on a decoction of strawberry leaves, bacteria with a pineapple aroma—upon a medium of pineapple leaves, bacteria with a radish-like odor—upon a medium of yellow radishes.

It has been further noticed that the same culture often changes its original pure fruity odor into an unpleasant odor. This can be explained in two different ways. We know that certain products of putrefaction, such as indol, give, in low concentrations, a very pleasant aroma. The presence of some indol in mixture with the ethereal oil of jasmine explains the wonderful aroma of this plant. In larger concentrations, indol possesses however a repulsive odor. The original pleasant odor in cultures of aroma-producing microörganisms may thus change into an unpleasant odor depending upon the concentration of aromatic substances.

But it is also possible that there is originally formed in the culture a pleasant aroma, which is later decomposed with the formation of substances of different odors, sometimes unpleasant ones. This is true of several aroma producing bacteria which

develop first in milk a fruity odor and later the characteristic sharp aroma of Swiss cheese. In some cases the reverse may take place: at first bad-smelling products are formed, later changing to aromatic products. This is true of *B. esterificans* which according to Maaszen develops in meat-pepton bouillon at first a strong odor of mercaptan, which later changes to a pineapple odor.

The group of aroma-producing microörganisms consists of representatives of various biological families. The most important of these are:

1. Yeasts. Aroma formation is the property of many cultivated and wild yeasts, especially those belonging to the genera *Mycoderma*, *Pichia*, *Willia* and partly *Torula*. These yeasts form on the surface of the nutritive substratum a dry pellicle or a mouldy growth, which produces a pleasant odor of complex esters of various flavors. However, an excessive development of pellicle-forming yeasts on the grape may give it too sharp an aroma, which spoils the fine bouquet of old wines.

A good medium for the development of pellicle-forming yeasts consists, according to Beijerinck, of a solution of ammonium acetate with some potassium phosphate. Lindner incubated at room temperature for this purpose moistened grains of barley placed in a large tube loosely plugged at both ends with cotton. The colonies of pellicle-forming yeasts developed on the surface of the grains in the form of beautiful rosettes, producing a fruity odor.

Discoveries of aromatic yeasts have been made at various times. Kayser separated in 1891 from pineapple juice a species of yeast producing on artificial media the pineapple aroma. Adametz and Wilckens reported in 1892 a series of experiments with a lactic yeast isolated by Adametz, *Sacch. lactis*, which forms, from lactose, ethyl alcohol, acetic acid and a corresponding complex ester. This species gave to butter a very pleasant aroma. Lafar and Will isolated from beer, in 1893 and 1899, pellicle-forming yeasts with fruity aromas. Similar discoveries were made later by various authors (Lindner, Klőcker, etc.).

Yeasts producing in culture a pear and pineapple-like odor

were found in 1902 by Bail on rotting leaves of rhubarb. A year later Mazé isolated from various cheeses yeasts fermenting lactose with the formation of a cheesy odor. Madame Bachinskaia-Raichenko isolated from Kumiss in 1911 two forms of yeasts which produce a pleasant wine aroma on malt and Kumiss media. The aroma producing property was lost on continued cultivation upon laboratory media.

In 1914, Edwards isolated 3 species of yeasts from North American cheese which produce on malt media a pineapple and strawberry odor.

The above references, which could be greatly increased, show that pellicle-forming yeasts are widely distributed in nature and that they may play an important part in the production of aroma in various products.

2. Acetic acid bacteria. On oxidizing ethyl alcohol to acetic acid these bacteria form as a by-product the ester of ethyl acetate. In factories where vinegar is obtained not from alcohol but from wine, as by the Orleans process, there are formed in addition to the above ester other aromatic substances, giving to the product a complex aroma much valued on the market.

3. Lactic acid bacteria. The aromatic bacteria belonging to this group play an important rôle in the bacteriology of milk and milk products, being among the causes of the specific aroma of milk products. Of the lactic acid bacteria the most energetic ester forming species are: the small streptococcus of Hagenberg and Weigmann, *Bac.* No. 18 Storch, *Lactobacillus lactis-acidi* Leichmann, *Bact. acidi-lactici* Hüppe, *B.* K. Weigmann, etc. However, several races of bacteria, bringing about a natural souring of milk, like *Lactob. lactis-acidi* and *Streptococcus Hollandicus* possess the same property of aroma formation, giving to milk and milk products a pleasant complex ester odor.

4. Butyric acid bacteria. The phenomenon of ester formation is especially noticeable in mixed cultures. Winogradski used such cultures for the growth of the anaerobic nitrogen-fixing organism *Clostridium Pasteurianum* belonging to the butyric acid bacteria. This species rapidly develops, in the presence of

oxygen, in a thin layer of liquid, due to a symbiotic development with aerobic species; the cultures thereby produced give off a complex-ester aroma resembling the odor of apples.

5. Putrefactive bacteria. The decomposition of proteins by means of bacteria is usually accompanied by the formation of evil smelling products of putrefactive decomposition. However, as Nencki properly pointed out in 1889, to characterize the action of the protein organisms it is not sufficient to know whether the odor of the products of decomposition is bad or good, but it is important to know the nature and degree of decomposition. Those species which decompose proteins with the formation of pleasant odors will therefore also belong to the putrefactive organisms. Numerous such microörganisms have been described, and we will later discuss in detail several of them. Species that play an important part in the ripening of cheese seem to belong here; the casein of the milk is decomposed with the formation of products of decomposition giving out a specific aroma.

6. Pathogenic bacteria. Cultures of some pathogenic bacteria, such as those of *Mycob. tuberculosis* and *Ps. pyocyanea* above mentioned, produce a pleasant aroma.

7. Mold fungi. Lindner isolated in 1889 a fungus *Sachsia suaveolens*, which occupies an intermediate place between yeasts and molds. It produces fermentation of malt liquids with the formation of a complex ester odor, resembling the odor of wine. This was the reason why Lindner called the fungus "Weinbouquet Schimmelpilz." An attempt was made to use this species technically to give a bouquet to non-alcoholic beverages. Mirsch and Eberhard took out a patent for the treatment of apple juice for ten days at 15 to 20°C. with this species till the surface of the liquid was covered with a pellicle of the mold and fermentation began. The liquid was then filtered and saturated with CO_2, producing a pleasant beverage with an aroma of Mosel-wine.

Several species of *Oidium lactis* impart to butter a nutty odor and a taste of mushrooms, while others give the odor of honey. The *Oidium suaveolens* isolated from water by Krzemeck develops a fruity aroma on malt media and on beer must. When growing upon sugar media it produces traces of ethyl alcohol and a small quantity of citric and malic acids.

Schnell isolated in 1912, from milk and milk products, several Oidia imparting to milk especially under acid conditions a more or less sharp odor and the taste of cheese. The author believes that they are normal agents in the ripening of cheese.

Several representatives of the genera *Penicillium*, *Mucor*, *Actinomyces*, etc. also produce a fruity odor, like *Penicillium aromaticum casei* I and II.

Kayser in 1891 and Went in 1893 isolated molds producing a pineapple odor. The latter isolated from Javan cane the fungus *Thielaviopsis aethaceticus*, which produces a pineapple odor in the presence of dextrin, sucrose and glucose. The fungus is a plant pathogen. Several Mucorales, like *M. piriformis*, *M. javanicus*, *M. mucedo*, *M. racemosus*, etc., impart to butter an odor of bitter almonds.

After this general reference to the complexity of the group of aroma-producing microörganisms, we may describe a few species in detail adhering to a chronological order.

The first reference to the presence in milk of bacteria which produce a fruity odor was made by Beijerinck. Milk placed in a shallow layer and coagulated by the rennetic enzyme produced after some time at room temperature a pleasant fruity aroma. By diluting and plating out on solid media, he succeeded in isolating a species possessing definite proteolytic properties at the same time producing an aroma; this species was called *Bact. aromaticum*. Weigmann isolated in 1890 a gelatin-liquefying, spore forming bacillus, *Aromabacillus Weigmanni*, imparting a fruity odor to milk. The same year Sclavo and Gosio described an aerobic spore-forming species, *B. suaveolens*, first hydrolyzing starch with the formation of dextrin and glucose, then decomposing it with the formation of esters of butyric and acetic acids. The same odor is produced by the growth of the organism in milk, meat extract and infusions of hay, straw and beet sugar. This species liquefies gelatin energetically.

Tatarov, in a Dissertation (Dorpat) on "Die Dorpater Wasserbakterien," described a weakly motile (one polar flagellum) bacillus ($1.5 \times 3.5-5\ \mu$) with a round spore, isolated from well water, *B. crassus-aromaticus* Tatarov (= *Pseudomonas aromatica*

Migula). This species forms on meat pepton agar a white, moist growth, with the development of a fruity odor. Gelatin is liquefied in a funnel-shaped manner giving a dirty deposit at the bottom.

In 1894, Henrici isolated from cheese several species giving a complex ester odor of various shades, gradually changing into a cheesy odor: a facultative anaerobic form, a motile bacillus (0.6 × 0.3 μ), allied to *Bact. aromaticum*, liquefying gelatin, *B. odorus*, forming a honey-like growth on meat pepton agar; *M. odorus* and *adoratus* (1.8 μ in diameter), etc.

The practical dairymen became greatly interested in the non-spore forming, non-motile bacillus (0.7 × 1.1 μ) often united in pairs and isolated by Conn in 1895 from South American (Uruguay) milk. This form is known as *Bacillus* No. 41 Conn and belongs to the *coli-aerogenes* group. Meat pepton bouillon is made turbid with the formation of a surface pellicle. On meat pepton gelatin, it grows better in the streak than on the surface. A shining white growth is formed on meat pepton agar. On inoculating milk with this species the acidity is first increased and a strong complex-ester odor is produced. Casein is then peptonized and the reaction becomes alkalin. The milk turns brownish and the aroma gradually becomes cheesy. When winter butter is inoculated with this species, it acquires the aroma of June butter, the "grass flavor," so highly prized on the market. However, not all investigators have obtained the same favorable results in the application of this species to practical butter making. This is probably due to the degeneration of the microbe and the loss of aroma-producing properties.

Pammel described in the same year a facultative anaerobic, motile, non-spore forming bacillus (0.3–0.45 × 0.9–1.2 μ) *Bact. aromaticum*, often united to form short chains, liquefying gelatin in a funnel-shaped manner and coagulating milk. Meat pepton bouillon is clouded with the formation of a yellow sediment. Growth on agar is at first white, then gray and finally brown. Glucose and sucrose are fermented with the formation of gas. In milk at first a pleasant aromatic odor ("butyl odor"), is produced which gradually changes to a stronger and distinctly

cheesy one, reminding one of the odor of Limburger cheese. Milk is coagulated and the coagulum is then gradually dissolved.

Bact. butyriaromafaciens isolated in 1897 by Keith also belongs to the acid-forming and gelatin-liquefying species; this is a non-motile, non-spore forming bacillus (0.5 × 1 μ) imparting to the milk a specific acid odor of butter. Milk is not coagulated. Bouillon is clouded with the formation of a sediment. An abundant white growth is formed on agar. Groups of two cells are often found in culture.

Burri isolated the same year, from 6 samples of Emmenthal cheese, the same species—a facultative anaerobic, motile bacillus (1.3–1.5 × 3–6 μ) with oval spores, named by Lehmann and Neumann *B. bernensis* and by Matsuschita *B. odoratus*. This species inoculated into agar forms fungus-like branching colonies. Gelatin is slowly liquefied in funnel shape. Bouillon becomes rapidly turbid with the formation of a pellicle on the surface and sediment on the bottom. Milk is coagulated in twenty-four hours at 30°. The coagulated casein is gradually dissolved, and the liquid becomes yellowish-brown, with the odor of Emmenthal cheese. The same odor is found to some extent in meat pepton media. Eckles isolated in 1898 from milk a non-motile aroma-forming bacillus (about 1.3 μ long) *Bac.* No. 13 Eckles, related to *Bacillus* No. 41 Conn.

In an extensive work published in 1899, Maaszen described 4 species, the cultures of which produce aromatic odors:

1. Bact. esterificans Stralauense isolated from the water of the river Spree (near Berlin). This is a rather large, motile, non-spore forming bacillus, allied to the intestinal bacteria. It produces a slight ester-odor on meat pepton media, rapidly lost on further transfer.

2. B. esterificans isolated from putrefactive litmus solution, a facultative anaerobic, peritrichic bacillus (0.5–0.6 × 1.4–5.2μ) with a large oval spore (1.2–1.4 × 2.7–3.2 μ). It produces in meat pepton media the odor of pineapple or fresh apples. Gelatin is not liquefied, with the formation along the streak of a granular white thread. On meat pepton bouillon at first a strong odor of mercaptan is formed and later a pineapple odor. The bouillon

is made very turbid with the formation of an abundant grayish-white deposit on the bottom. Milk is slowly coagulated (negative according to Husz) with the formation of a characteristic pineapple odor. The aroma-producing property is not lost by this species for a year.

3. Bact. esterificans fluorescens was found by Maaszen on grains, and on rotting plants in river water. This is a small, non-spore forming motile bacillus, producing in cultures a fruity odor, gradually changing to that of trimethylamine. Gelatin is not liquefied.

4. Bact. praepollens was isolated from the sweat of one sick with cholera. This is a non-spore forming non-motile bacillus, rapidly liquefying meat pepton, gelatin, blood serum and milk casein. It decomposes urea rapidly and can reduce nitrates. In the decomposition of pepton and growth on milk, there is produced an odor of amylvalerianic ester, especially in the presence of abundant aeration. Later, the odor loses its purity and gradually changes to one of ammonia and cheese. The property of aroma formation is kept for years. Carbohydrates are decomposed without the formation of gaseous products.

In 1899, Weiss isolated from turnips an aroma-forming bacillus, *Bact. odoratum*. In 1900, Reinmann isolated from bitter butter a motile bacterium belonging to the group of intestinal bacilli, or soil bacilli. On inoculating fresh butter with this species, a strawberry aroma is soon produced. The following year Glage isolated from common manure a short bacillus related to the intestinal bacteria but liquefying gelatin with a fruity odor.

Rullmann isolated in 1902 from milk a spore-forming, non-motile, fluorescent species, producing in cultures a fruity odor. Size 0.4 × 1.5 μ. Spores-terminal. Gelatin is not liquefied. It belongs to the facultative anaerobes.

The same year (1902) appeared the work of Grimm, Eichholz and Grüber. The first isolated from milk a very motile, peritrichic bacillus (0.8 × 3.5 μ) *B. aromaticus lactis*, belonging to the facultative anaerobes. This bacillus forms in culture a great deal of slime and produces a complex ester odor, gradually changing to a cheesy one. A dirty-gray slime appears on meat

pepton agar. Gelatin is not liquefied, with the formation of pearl-like growth along the streak. Milk becomes slightly sour but is not coagulated; the milk gradually becomes brownish and a strong fruity odor develops due to the formation of ethyl lactate. After four to five weeks, the milk is coagulated with the formation of a cheesy odor. The coagulated casein is weakly peptonized.

Eichholz isolated from milk a monotrichic bacillus (1.05 × 1.75–2.10 μ), allied to the intestinal groups *Bact. fragi* (*Pseudomonas fragi*), producing on meat pepton agar at first a strawberry odor, then that of methylamine. The colonies of this species on lactose-gelatin are characteristic; showing a granular central portion with scallop-like edges in the form of a rosette. Gelatin is not liquefied. Milk is not coagulated, the reaction changes to alkalin and a rotting odor is formed.

Grüber isolated from river water a motile, non-spore forming bacillus *Pseudomonas fragariae* I., which produces a fluorescence in media. Bouillon is made turbid with a slimy sediment. The reaction becomes distinctly alkalin. Gelatin is not liquefied, growth is on the surface and not along the stab. On meat pepton media, the odor is at first that of strawberry and pineapple, in time changing into the ammoniacal odor of decomposing urea. A peculiar odor and bitter taste is imparted to milk. In a month the milk becomes brownish. Sterilized milk inoculated with this species produces after some time a strawberry odor.

In 1904, Severin isolated from cream a motile non-spore forming bacillus *Bact. aromaticus butyri*, producing on meat pepton media at first a fruity odor, then a rotting ammoniacal odor. Gelatin is not liquefied and milk is not coagulated. The fruity aroma is produced on milk only when lactic acid bacteria are also present, and is especially prominent during the period previous to milk coagulation. When added to fresh butter, this species imparts to it in a month a fruity aroma. The aroma producing property is not lost in eight months.

Gaethgen isolated in 1905 from secretions of individuals suspected of typhus, a non-spore forming, weakly motile bacillus, producing a strong fruity odor in milk, *Bact. flavo-aromaticum*,

forming yellow colored colonies. By its culture and odor formation in culture, this species is related to *B. crassus aromaticus* Tatarov. Gelatin is not liquefied.

Grüber isolated the same year, from pasteurized milk, a motile bacillus, *Pseudomonas fragariae* II, producing a marked odor of strawberries on meat pepton media, especially gelatin, which is weakly liquefied on the surface.

Beck described in 1906 an acid-forming *Micrococcus esterificans*, developing a complex ester fruity odor. This species acts very weakly on proteins.

Huss described in 1907 two species producing a fruity aroma: *Bact. esterificans* and *Pseudomonas Trifolii*. The first species was first isolated by Maaszen, but Huss described it in detail. The second was found on clover. This is a monotrichic, non-spore forming bacillus (0.5–0.7 × 0.75–2.1 μ), growing on agar in the form of a transparent gray-yellow growth, remembling a layer of vaselin. It liquefies gelatin and decomposes proteins, with the formation of H_2S. On media containing carbohydrates and proteins, the carbohydrates are first decomposed and the liquid changes from acid to alkalin. Milk is coagulated in two weeks by means of rennet, with an odor of fresh hay. The coagulated casein gradually dissolves, and the milk develops a bitter taste. In another article published the same year, Huss described a motile non-spore forming bacillus (0.6 × 1–4 μ), *Peudomonas fragaroidea*, developing in cultures first a strawberry, then an ammoniacal odor On meat pepton bouillon and agar, fluorescence and a strong alkalin reaction are produced. Gelatin is rapidly liquefied. Milk is coagulated without change in reaction and the coagulum is gradually dissolved. The original strawberry odor changes to pineapple.

In 1907 there also appeared an article by van der Leck, describing two microbes with a fruity odor:

1. Bact. aromaticum, a very motile bacillus (0.8 × 2.5–3.5 μ); allied to *Bac.* No. 41 Conn. It forms yellowish slimy colonies on gelatin, slowly liquefying it around the colony with the formation of an odor. A dirty-white slimy growth on agar. Under the influence of this microbe the milk obtains a peculiar, acidic

aroma with very weak acidity; it coagulates very slowly and turns gradually brown. Of the carbohydrates, only glucose is utilized. The optimum temperature for growth is 23°C.

2. Bact. acido-aromaticum, a non-motile and gelatin non-liquefying bacillus of the same size as the previous form. It makes the milk quickly acid with the formation of a butyric acid odor.

For the accumulation in culture and separation of aroma-producing bacteria, van der Leck used the method first introduced by Beijerinck. Market milk is warmed to 30°C. and, adding some rennet, is poured in a thin layer into a Petri dish. The milk is coagulated at 20 to 23° and usually begins to produce in twenty-four hours a fruity odor. A gelatin medium prepared with milk whey is used for isolation.

Kurono isolated in 1911 two aroma-producing races of *B. butyricus* from the rice-wine "Saké-Moromi." By fermenting glucose with these species an ethyl butyric ester is formed.

The above list of aroma-producing microbes is far from complete and cannot even pretend to be, particularly since many sources remained unattainable for the author, especially during the last few years. Nevertheless, even on the basis of the material presented, a definite idea can be obtained of the great variety of this group.

Among the sources from which the organisms were isolated, we find milk products, water, excreta, etc. However, the source of isolation cannot serve any purpose for explaining the peculiarities of the isolated microbe. This explanation can be given only in special cases. For example, the microbes isolated from milk develop in artificial cultures products having a cheesy odor.

The same lack of uniformity is also found in the morphological characters of the organisms. We find here cocci, short and long bacilli, spore forms and non-spore forms, motile and non-motile forms. A large number of the species belong to the coli-aerogenes group or are closely related to them.

There is also a great difference between the various species in the type of odor and constancy of aroma-production, which is rapidly lost for many species in cultivation on artificial media.

Only a few general features are recognized here. For example, bacteria originally producing a very fine strawberry aroma later usually change it into a sharp ammoniacal odor, that of putrefying urine or a specific putrefactive odor, as in the case of *Bact. fragi* Eichholz, *Pseudomonas fragariae* I Gruber and *Pseudomonas fragaroidae* Huss.

Attention has been already called to the variety of biochemical characteristics of the aroma-producing microbes. Attention must be drawn here to the fact that some species were described so superficially, that it is impossible to get a clear idea of the chemistry of their processes. The great number of organisms capable of decomposing proteins is marked: *Bact. aromaticum* Beijerinck, *B. crassus aromaticus*, *B. odorus*, *Bac.* No. 41, *B. bernensis*, *Bact. praepollens*, *Bact. fragi*, *Bact. fragariae*, etc.

This makes possible the hypothesis that the main cause of aroma-formation in wine, milk and milk products is the production of odoriferous products from the decomposition of proteins.

This group of microörganisms has been attracting, for the last few years, more and more attention since to the species belonging here is ascribed an important part in the natural production of aroma in various food products, especially milk products—butter, cheese, curdled milk, milk, etc. Their rôle in imparting a bouquet to various wines of grape, berries and fruit, as well as beer is also possible.

This investigation is devoted to the description of several new species of aroma-producing microbes.

The question of aroma-producing bacteria came up twice, in my laboratory experience, and both times accidentally, in 1897 and 1915.

In the spring of 1897, when working with cellulose-decomposing bacteria, an experiment was started dealing with the decomposition of pieces of raw potato in an open beaker filled with tap water. An active fermentation took place accompanied by gas production, and the potato gradually decomposed and became transformed into a starchy mass. The turbid liquid above the rotted material gave out a repulsive odor. Several dilutions

were made from this upon meat pepton agar. After some time at 35°C. the cultures in the Petri dishes began to produce a pleasant fruity aroma resembling the odor of apples. By transferring the minute colonies developing upon the agar, a pure culture was obtained of the organism causing this odor. It proved to be a non-characteristic, short, non-spore-forming bacillus, belonging to the protein decomposing bacteria and entirely inactive in regard to the carbohydrates. After a few preliminary experiments the work had to be stopped due to my leaving Petrograd for the summer. When the work was resumed it was found, to my great regret, that the culture had died during the summer and could not be revived. Attempts to reisolate the culture using the previous method proved to be also in vain.

Eighteen years later, in the fall of 1915, I succeeded in isolating the organism again. It is known that in the preparation of anti-rabies virus the brain of the rabbit used as virus is first tested for sterility. For that purpose a piece of it is placed in a tube with sterile bouillon and the latter is incubated at 37°C. If the bouillon becomes turbid and bacteria multiply in it, the brain tissue cannot be used. Such a case, which is a comparatively rare one, took place in August, 1915 in the Inoculation Department of the Institute of Experimental Medicine, the turbid bouillon producing a fruity aroma. The tube with the bouillon was kindly given to me by Dr. R. G. Pirone. Most unexpectedly the same organisms which I had possessed many years ago developed in the tube. Again, I succeeded in obtaining rapidly a pure culture without any difficulties by merely diluting on meat pepton agar.

Before describing this species I must remar kthat its original habitat remains unknown to me, since both times the organism was found by accident, and attempts to obtain it from raw materials gave no results. But judging by the repeated occurrence of this organism one may conclude that this species is widely distributed in nature.

Like the majority of described bacteria producing a fruity odor, the species that I have isolated belongs to the small, non-spore forming bacteria, closely related in appearance to the intestinal bacillus. Its dimensions are 0.5 × 1–3 μ. By ex-

amining an emulsion of live bacteria in a drop of water, no active motility was observed. Attempts to demonstrate flagella by means of various processes of staining also remained unsuccessful. This species does not produce any slimy capsules or zooglea formations and is, in general, very poor in distinguishing characteristics. Yet since, by biochemical characteristics, it cannot be identified with any of the previously described forms, it is suggested that it be recognized as an independent form, calling it, after its most distinguished character, that of forming aromatic products—*Bact. esteroaromaticum*. It belongs to the facultative anaerobes, growing better, however, with the admission of air.

This bacterium possesses a distinct reducing action. A bouillon culture in a tube colored with a solution of methylene blue is decolorized within one minute. By shaking up the liquid, the color again returns. A suspension of bacteria obtained from an agar slant in water does not possess this reducing property. This leads us to think that the reduction is a property of some products of metabolism dissolving in the bouillon. The decolorization of indigo carmin and neutral red took place just as rapidly.

Arsenates added in concentrations of 0.001 to 0.02 per cent to the medium are not reduced: no garlic odor is produced by the cultures. By inoculating a slant of meat pepton agar to which 2 drops of a 2 per cent potassium tellurate[1] is added, the bacterial growth became blackened on the second day, especially near the water of condensation.

Meat pepton bouillon becomes rapidly turbid, especially in the upper layers. At 25° there is produced on the third day a faint fruity aroma, gradually becoming more pronounced but losing in purity. This odor with a definite flavor of esters of valerianic acid, reminds one of the aroma of apples, changing in degree depending on the composition of the medium and conditions of cultivation. By inoculating meat pepton gelatin, cup-like liquefaction begins at room temperature on the second day. In a week, the *depth* of the liquefied portion is 1.5 cm. and the culture produces an odor resembling that of musk-melons.

[1] Sodium tellurate commonly used could not be obtained in the laboratory.

On meat pepton agar at 35° there is produced a fruity aroma on the second day and the medium becomes slightly phosphorescent. The colonies are of a yellow-brown color with slight festoon-like edges. On agar containing 1 per cent glucose the odor is weaker and not so pure as on plain agar. The addition of a few drops of ethyl alcohol to meat pepton agar notably increases aroma-formation. Fluorescence is more pronounced on this medium. Meat pepton agar with 6 per cent glycerol is less adapted to the development of *Bact. esteroaromaticum*, although a strong aroma is produced with a flavor of the musk-melon odor.

Heyden's agar is little adapted to the development of the organism, but even on this medium an aroma is produced. Only a weak growth without any odor formation takes place on malt agar.

The growth of the microbe in milk is characteristic. During the first few days the milk produces the repellent odor of a lard candle, due to the decomposition of the fat. The casein dissolves gradually, starting from the surface, the milk becoming light brown. Milk free from fat produces from the beginning a pleasant aroma, recalling that of valerianic ester. This odor changes finally to the characteristic aroma of old cheese; especially when the milk is made slightly acid. The addition of 0.5 to 1 per cent pepton stimulates the growth of the organism and its aroma formation.

Growth on potato is abundant, but the odor is unpleasant. A three per cent pepton water becomes slightly turbid with the development of a light fruity odor.

A strong and pure aroma of a complex ester is produced, on the third day, on a medium of the following composition:

Tap water	1000 cc.
Pepton	5–10 gm.
Potassium phosphate	1 gm.
Agar-agar	20 gm.

especially if a few drops of ethyl alcohol are added.

A streak on an ox-serum slant shows on the second day, liquefaction along the streak, which becomes deeper, while the lique-

fied serum flows down in the form of a brown mass. All the serum is liquefied on the fifth day with the production of a fruity aroma.

On the basis of the above data one may conclude that *Bact. esteroaromaticum* belongs to the protein decomposing organisms, growing best on pepton media. The addition of propyl alcohol to the medium has no influence on the strength or quality of the odor. Amyl alcohol possesses by itself such a strong aroma that it represses the fruity aroma of the culture. The addition of mannitol has a depressing effect upon aroma formation in pepton media. The increase in concentration of pepton to 2 per cent stimulates the formation of aromatic substances.

A fair growth and a pleasant fruity aroma with a peculiar flavor is found in media containing egg or blood albumen as in the case of the following medium:

Distilled water	1000 cc.
Egg albumen (Kahlbaum)	50 gm.
Potassium phosphate	1 gm.
Magnesium sulfate	0.5 gm.
Sodium chloride	0.1 gm.

The egg albumen is first dissolved in a warm solution, and the liquid is placed in 50 cc. portions in Winogradski flasks. After sterilization the albumen solution is precipitated in the form of a solid white mass. It begins to liquefy on the second day, and in a week all the albumen is dissolved. The culture produces a strong aroma with a characteristic flavor. On gelatin, wool, nutrose, aleuron, gliadin and keratin, no growth or aroma formation takes place. *Bact. esteroaromaticum* acts upon casein (nach Hammersten-Merck) only in the presence of soda: 0.1 gram per 1 gram of casein. Under these conditions, casein is readily dissolved with the formation of the complex ester aroma.

As pointed out above, milk casein as well as casein precipitated by rennet is rapidly dissolved. But the casein from sour milk is not acted upon due to the reaction of the medium.

The organism develops readily with the formation of a fruity aroma on "vegetable casein" or legumin. The organism acts energetically on fish protein prepared by Prof. M. D. Ilün and

kindly placed at our disposal for investigation. The protein mass, grayish in color, was faintly acid and, for the favorable action of the microbe, it had to be neutralized (1 cc. of a 5 per cent solution of soda to 5 gram of protein). In a few days, after inoculation at 30°, peptonization of the mass and odor formation took place. Of the amino acids (and acid amides), alanin, asparagin and urea are unsuitable for the development of *Bact. esteroaromaticum* alone and in the presence of carbohydrates. Weak growth takes place upon 1 per cent solution of leucin and α-amino-isocaproic acid. The addition of leucin to meat pepton bouillon or to pepton solution stimulated the growth of the organism and the formation of valerianic ester, especially in the presence of ethyl alcohol.

The mechanism of ester formation and the intermediary stages through which the protein passes when it is decomposed by the organism are of great interest. Unfortunately, one can answer the question only by indirect considerations. In view of the fact that in the decomposition of proteins by *Bact. esteroaromaticum* esters of valerianic acid are formed, it appears that proteins are decomposed with the splitting of leucin or iso-leucin and the latter is further decomposed with the formation of valerianic acid, amyl alcohol and compounds of the ester type, as iso-amyl-valerianic ester (found by Maaszen in the decomposition of proteins by *Bact. praepollens*). The esters are probably formed by the interaction of the alcohols and acids.

The formation of optically inactive iso-amyl alcohol from *l*-leucin takes place according to the equation:

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CH \cdot CH_2 \cdot CH\,(NH_2) \cdot COOH + H_2O =$$

leucin

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CH \cdot CH_2 \cdot CH_2\,(OH) + CO_2 + NH_3$$

iso amyl alcohol

Iso-leucin is transformed in an analogous way into optically active *d*-amyl alcohol, by the splitting off of CO_2. This transformation has been observed by F. Ehrlich in the decomposition of leucin by yeasts and by Pringsheim for molds.

This method is the more probable, since in the decomposition of proteins by *Bact. esteroaromaticum* an odor of apples is produced, particularly strong in the presence of ethyl alcohol, an odor, as is well known, determined by the ester of iso-amyl-isovalerianic acid. This ester mixed with 5 to 6 parts of ethyl alcohol is used for obtaining the artificial "apple ester."

In old cultures the ester aroma passes gradually into a cheesy odor, especially noticeable in milk. It is known that one of the causes which determine the odor of old ("rotten") cheese is the presence among the products of decomposition of isovalerianic acid. As will be seen later, there are direct indications of the formation of valerianic acid in the decomposition of proteins by our microbe. All these considerations make us recognize as entirely probable the supposition concerning the mechanism of decomposition of proteins by *Bact. esteroaromaticum* with the formation as intermediate products of leucin and iso-leucin and from these, various compounds of valerianic acid.

As seen above, free ammonia is formed in the decomposition of leucin. Considerable quantities of ammonia could be demonstrated in the cultures from the decomposition of proteins. The ammonia is determined qualitatively and quantitatively by distilling with magnesium oxide and titration of the $\frac{N}{10}$ sulfuric acid.

Among the products of decomposition of the proteins, the presence of H_2S was demonstrated. A piece of paper moistened with lead acetate solution and placed above the culture of *Bact. esteroaromaticum* darkened on the second day. On agar with lead acetate, the colonies became brown.

Neither indol nor tryptophane were demonstrated in two week old pepton cultures. Weyl's reaction for creatinin gave positive results.

To obtain a more definite measure of the degree of decomposition of different proteins by our organism the amino nitrogen was determined by the method of Sörensen. One hundred cubic centimeter portions of the mineral medium were placed in 5 Winogradski flasks and 1-gram portions of the following proteins were added to the different flasks: egg albumen (Kahlbaum),

blood albumen (Kahlbaum), pepton (Okunev), casein nach Hammarsten (Merck) and keratin-Zyzkia (Merck).

The flasks were kept at 30 to 31° for two weeks. The results are as follows:

KIND OF PROTEIN	DEVELOPMENT OF ORGANISM	DEGREE OF AROMA-FORMATION	AMINO-NITROGEN
			mgm.
Egg albumen	Very good*	Pure fruity aroma	47.6
Blood albumen	Very good*	Odor not so pure	47.6
Peptone	Very good*	Odor of ammonia	43.4
Casein	No growth	No odor	5.6
Keratin	No growth	No odor	5.6

* Rapid peptonization.

In another experiment, 5 per cent egg albumen (Kahlbaum) was added to the mineral solution, and 50-cc. portions of the liquid containing 2.5 gram albumen each were placed in 20 Winogradski flasks. Three drops of a suspension of *Bact. esteroaromaticum* grown on agar were added to each flask.

On the second day, orange-yellow spots appeared on the surface of the coagulated albumen at the places of inoculation and the culture began to produce a fruity odor with the flavor of valerianic ester. The peptonization of the albumen was noticeable on the third day. The amino nitrogen was determined in one flask on the fourth day, according to Sörensen, and it was found to be 44.8 mgm. to 1 gram of albumen.

Since energetic peptonization was observed in all the flasks, it was thought of interest to determine the content of proteolytic enzyme. The determination was carried out according to the methods of Fuld-Gross and Fermi.[2] Two hundred cubic centimeters of water were added to the contents of one of the flasks to make a 1 per cent solution of the original protein. The turbid liquid was filtered through a paper filter and the filtrate distributed in 10 tubes in decreasing quantities (cubic cm.): 1, 0.5, 0.25, 0.125, etc., i.e., in decreasing geometrical ratio. By the

[2] Wohlgemuth, Grundriss der Fermentmethoden, 1913.

Fuld-Gross method, after one hour's action at 38°, a complete dissolution of the casein was obtained only in the first tube (1 cc.) and partial dissolution in the second, with complete liquefaction of the gelatin in the first tube according to the method of Fermi.

On the seventeenth day after inoculation, the contents of all the flasks were poured together for a general analysis. A microscopic examination of the liquid indicated that the culture remained uncontaminated. The thick yellowish-brown liquid possessed an apple aroma with a flavor partly of valerianic ester and partly of ammonia. The reaction of the liquid was distinctly alkalin. The following analysis were made: total nitrogen according to Kjeldahl, amino-nitrogen by the method of Sörensen, peptide nitrogen by the method of Henriques-Sörensen and ammoniacal nitrogen by the method of Spiro. In addition to this the following protein color reactions were made: biuret, Millon, Liebermann, Adamkiewicz, Keanto-protein, Molish-Udranovski, Pettenkoffer and lead acetate.

For analysis there were used: I, liquid of original culture; II, same liquid, made slightly acid with acetic acid and filtered after boiling; and III, second liquid boiled with animal charcoal.

All color reactions gave good results with the first liquid, but were weak with the second and negative with the third. The following table gives the various nitrogen determinations, on the basis of 1 gram original protein, in milligrams.

	I	II	III
Total-N	122.5	71.8	39.1
Amino-N	53.2	46.0	34.5
Peptide-N			34.5
Ammonia-N			1.18

The composition of the volatile fatty acids was determined, according to Duclaux, in a separate experiment. The liquid, slightly acidified by means of oxalic acid, was distilled three times with the following results:

FRACTION	$\frac{N}{20}$ NaOH	PER CENT	THEORETICAL AMOUNT OF MIXTURE 1 PART ACETIC AND 1 PART VALERIANIC ACIDS
	cc.		
1	11.2	18.8	
2	20.0	33.5	34.1
3	27.5	46.1	46.4
4	33.9	56.7	64.7
5	39.4	66.0	64.7
6	44.1	74.0	77.0
7	48.3	80.9	78.7
8	52.2	87.4	
9	55.8	93.4	
10	59.7	100.0	

The correlation is not very perfect, but the discrepancies among the figures can be explained by the admixture of other acids, to which indications seemed to point. For example, the solution of volatile acids gave the reaction with silver nitrate (admixture of formic acid).

Bact. esteroaromaticum does not attack carbohydrates, higher alcohols and salts of organic acids. Among the substances tested were: glucose, sucrose and lactose, dextrin, inulin, starch, gum-arabic, dulcitol, mannitol and ammonium tartrate. All these compounds were of no use for the cultivation of the organism in the absence of proteins.

It has been already stated that the organism hydrolyzes milk fat. It has the same action on fish fat added to meat pepton agar. It does not act on sunflower oil under the same conditions.

Tests for denitrification gave negative results: not only was no free nitrogen produced, but there was even no reduction of nitrates to nitrites.

On the basis of the above data, we may conclude that this organism belongs to the typical protein bacteria, readily growing on media with egg and blood albumen, on casein (in the presence of soda), pepton, etc. All these substances form sources of both nitrogen and carbon. The property of aroma formation distinguishes our organism from the morphologically related common species; as to the aroma producing forms, none is identical with

it. It is most closely related to *Bact. praepollens* Maaszen, but the latter is capable of denitrifying nitrates and decomposing urea.

Some brief observations during this work indicated the fact that the degree and character of aroma formation may change in the presence of other species. In one case the fruity odor was markedly increased in symbiosis of *Bact. esteroaromaticum* with a large, non-spore forming bacillus which was isolated from mud and fermented various carbohydrates energetically. The influence of this species was particularly marked in sugar media. The co-action with other species has not been studied in greater detail, although this question is of great interest.

The idea naturally suggested itself of utilizing the isolated species for practical purposes, namely, to give an aroma to various milk products, particularly cheese. Until now the application of aroma-forming bacteria to practical purposes has been difficult due to the fact that they rapidly lost the property of producing aromatic products when cultivated in the laboratory on artificial media. This physiological degeneration doubtless explains the lack of correlation between the results obtained by various authors for the same species. While Bacillus No. 41 gave excellent results in the hands of Conn and other American investigators, Farrington and Russell could not get any results at all. Another example is *B. nobilis* isolated by Adametz and Kleck and recommended by them for increasing the aroma of cheese. Dried preparations of this culture were placed on the market under the name of "Tyrogen." However, the cheese prepared by Freudenreich and Troili-Peterson, by means of this culture, was of a poorer quality than the control, doubtless due to the degeneration of the species and loss of original properties.

In the light of these and similar facts, the ability of *Bact. esteroaromaticum* to preserve for several years, without noticeable weakening, the property o faroma formation is of special importance. It is three and one-half years since the microbe was isolated and it still preserves, almost completely, its properties.

The capacity of *Bact. esteroaromaticum* to hydrolyze fats made us give up early the idea of adding aroma to butter. However,

various facts were in favor of its application to the ripening of cheeses, especially those prepared from skimmed milk. One would think that, in addition to its adding a proper aroma to the cheese this species might partially hydrolyze the casein and impart the proper consistency to the milk, which is very important for heavy cheeses.

Unfortunately, the experiments performed up to the present did not give satisfactory results due to purely accidental causes.[3] In The Nadezshdin Dairy School (Tambov Gov.) the culture was added 5–10 minutes before cooking and the chemical action of the organism did not become pronounced. Neither an aromatic nor a cheesy odor were produced. The prepared cheeses, Holland and Tilzit, were naturally only to a slight degree different from the controls. The results of the application of the culture of our organism in the Dairy Station in the Tuga Uyezd, Petrograd Gov., cannot be related as both the experimental and control cheeses were stolen from the cellar. The cheeses prepared in the third Station were forwarded to the address of A. A. Kalantar, but were requisitioned on the way and have not reached their destination. Unexpected results were obtained, according to C. A. Korolev, at the Lotoshin Experiment Station, where the experimental cheese swelled up and burst open. If this was due to gas formation, the organism in question is surely not the cause, since it produces no gas in culture. This phenomenon was not observed in the other stations.

It would be very desirable to repeat the experiments in the coming spring campaign of cheese making. The following experiments suggest themselves. Fresh milk is separated from fat and is abundantly inoculated with a culture of *Bact. esteromaticum*. The milk is kept at 30 to 35° for several periods to determine the optimum, at the end of which it is placed in the cellar for various periods. It seems that by the proper use of the culture the period of ripening could be greatly diminished without loss of flavor and plasticity.

[3] The author takes here the opportunity of expressing his sincere thanks to the coöperation rendered by A. A. Kalantar, member of Scientific Committee of the previous Ministry of Agriculture; O. I. Ivashkevitsch, Director of the Nadezshdin Dairy School; and to C. A. Korolev, Director of the Moskau Bacteriologo-Agronomical Station.

It is possible that the organism will find application in the leather industry—in the process of tanning especially in softening the skins.

These are only possibilities, since under the present conditions it is difficult, almost impossible, to conduct large technical experiments.

I isolated another organism from milk in February, 1918, by the method of Beijerinck and van der Leck. One hundred cubic centimeters of good milk received from the Milk Section of the Central Food Department were placed in a Winogradski flask and coagulated by means of rennet (Lab powder 1 = 300,000 Witte). In twenty-four hours, at 18°C., the cultures began to produce a light strawberry aroma, becoming much stronger in another twenty-four hours. Dilutions were prepared from this culture using meat pepton agar containing 1 per cent glucose and a pure culture was easily obtained of the organism producing the same aroma. This proved to be a non-motile, non-spore forming, aerobic bacillus, $0.8 \times 1.2\text{–}2\mu$, with small capsules around the cells seen in the India-ink preparation.

Meat pepton bouillon becomes uniformly turbid on the second day, forming a small sediment and developing a light strawberry aroma. In a few days a pellicle is formed on the surface of the bouillon and the odor becomes sharper, gradually changing into an aroma recalling that of cheese.

On meat pepton agar, an abundant white slimy growth appears along the streak on the second day. The culture produces a slight strawberry odor, remaining practically unchanged for three weeks. Growth on agar containing sugar is abundant, but no odor is formed. The latter appears only after two weeks, recalling the aroma of cheese. Meat pepton gelatin is gradually liquefied producing a fruity aroma. On potatoes, there is at first a brownish-rosy growth practically without any odor, then the growth becomes of a more pronounced rose color, with the development of an excellent strawberry aroma.

The milk is coagulated in four days with a slight strawberry aroma. On titrating with $\frac{N}{10}$ sodium hydroxide, it gave on the

fifth day 21° Thörner. We can thus conclude that coagulation takes place not by means of an acid but by lab ferment.

This species belongs to the protein decomposing organisms, growing very well on solutions of pepton, egg albumen, casein and other proteins. In many cases, however, an impure fruity aroma develops with an admixture of other flavors and sometimes this is quite unpleasant. An odor of hay develops in solutions of serum albumen.

The experiments with this species were soon stopped, since the culture completely lost the property of aroma formation and all attempts to renew it ended fruitlessly.

It is known that pressed yeasts, when kept for a long time, begin to produce a sharp complex-ester odor. We observed also such an instance on bread Kvas kept for a long time, on the surface of which a "mold" appeared; and then began to produce a fruity aroma. Mrs. O. C. Manoilov and C. U. Buchbinder studying in my laboratory the question of bread fermentation isolated several species of pellicle yeasts from various materials: (1) from market bread Kvas, (2) from a sample of bread yeast received from Zarskoselski yesd, Petrograd. Gov., and (3) from bread yeast received from Lifland.

All these pellicle yeasts, little distinguished morphologically and belonging to the genus Mycoderma, possessed almost the same physiological properties. To grow well on sugar solutions, they required an abundance of oxygen. The cultures were usually kept on media of the following composition:

Tap water	1000 cc.
Glucose	100 gm.
Ammonium phosphate	2 gm.
Potassium chloride	0.1 gm.
Magnesium sulfate	0.1 gm.

The solution is placed in a thin layer in Winogradski flasks and after sterilization inoculated with pure cultures of mycodermae. A pellicle appeared usually in two days at 30 to 35° on the surface of the liquid and the culture produced a thin sourish aroma, reminding one of the odor of table wine. No fermentation took place. On microscopical examination the characteristic cells

of pellicle yeasts were found with two vacuoles and droplets of fat in the opposite ends. These yeasts did not grow in media with cane sugar. This is characteristic of pellicle yeasts. As is well known, this property suggested (Beijerinck) the purification of cane sugar from glucose. No growth took place in solutions of galactose, lactose, maltose, dextrin, starch, gum-arabic and mannitol.

The observation was made that the fruity aroma produced in media with glucose was increased on adding 1 to 2 per cent ethyl alcohol and a few drops of acetic acid. The fact that mycodermae cannot utilize pepton, asparagin and other organic nitrogen compounds as well as ammonium salts and also the need of large quantities of carbohydrates so that a good growth should take place indicate that the sources of ester formation are in this case not the proteins, as in the above described bacteria, but the carbohydrates. Alcohols and organic acids are produced in the decomposition of the carbohydrates and form, *in statu nascendi*, the complex compounds.

In conclusion, we may mention one aroma producing mold isolated from a moldy lemon. This mold belonging to the species Penicillium, produced a fruity aroma of a peculiar flavor, recalling that of a musk-melon, only on media of a certain definite composition, as in bean decoction (Mazé) to which 5 to 10 per cent of maltose is added. When maltose is replaced by starch or inulin, an unpleasant odor develops on the same medium. Finally, with some sugars, the odor is not produced at all.